BRAIN ANGIOTENSIN AND THE NUCLEUS TRACTUS SOLITARIUS IN THE CENTRAL CONTROL OF BLOOD PRESSURE

BY

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To my parents.

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KEY TO ABBREVIATIONS

ACE	angiotensin converting enzyme
ANG	angiotensin
ANOVA	analysis of variance
AP	area postrema
Arg	arginine
AVP	arginine vasopressin
CSF	cerebrospinal fluid
DMV	dorsal motor nucleus of the vagus
g	gram
h	hour
HR	heart rate
IML	intermediolateral cell column
ip	intraperitoneal
iv	intravenous
kg	kilogram
MAP	mean arterial pressure
mg	milligram
min	minutes
ml	milliliter
mm	millimeter
mmHg	millimeters of mercury

ng	nanogram
nmole	nanomole
NTS	nucleus tractus solitarius
OVLT	organum vasculosum of the lamina terminalis
P	level of statistical significance
pH	negative log of hydrogen ion concentration
PI	pulse interval
pmole	picomole
PVN	paraventricular nucleus
s	seconds
SD	Sprague-Dawley rats
SE	standard error
SFO	subfornical organ
SHR	spontaneously hypertensive rats
SON	supraoptic nucleus
SYS	systolic arterial pressure
Tyr	tyrosine
ug	microgram
ul	microliter
um	micrometer
VIP	vasoactive intestinal peptide
WKY	Wistar-Kyoto rats

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Angiotensin II is a circulating hormone having profound effects on blood pressure, vasopressin secretion, and water balance. The discovery of this peptide in areas of the brain involved in central cardiovascular control has led to the hypothesized existence of a central renin-angiotensin system in the regulation of blood pressure. The nucleus tractus solitarius contains receptors for angiotensin II and immunoreactive angiotensin II has been localized there. As the central site for integration of baroreceptor function, the nucleus tractus solitarius represents a likely site for brain angiotensin to influence cardiovascular regulation.

The blood pressure and heart rate responses to angiotensin II were studied by direct microinjection into the nucleus tractus solitarius of rats. Angiotensin II increased arterial pressure and heart rate in a dose-dependent fashion. The pressor effect is mediated solely through activation of the sympathetic nervous system and is not dependent on the release of vasopressin as occurs with other routes of administration.

The central pressor response to angiotensin II is localized to the nucleus tractus solitarius within the brain stem. Several other neuropeptides localized in the nucleus tractus solitarius possessed no cardiovascular actions within the same dose range as angiotensin II. Only vasopressin and angiotensin II possess pressor effects when microinjected into the nucleus tractus solitarius. A possible relationship between these effects was investigated and each response proved to be independent of the other. The spontaneously hypertensive rat exhibited pressor responses to angiotensin II which were exaggerated compared to normotensive controls.

The possibility that angiotensin II acts at the nucleus tractus solitarius to modulate baroreflex function was studied. Subpressor infusions of angiotensin II into this nucleus decreased baroreflex sensitivity as measured by reflex slowing of heart rate to graded doses of phenylephrine given intravenously. These data enhance the existing evidence suggesting that brain angiotensin is important in central cardiovascular regulation by demonstrating a physiological function for this peptide in the medullary control of the sympathetic nervous system.

CHAPTER 1
BACKGROUND

Central Control of Circulation

The cardiovascular system is controlled by mechanical, chemical, and neural mechanisms whose contribution to circulatory regulation varies with the physiological state of the animal. Central control of the circulation is complex, as the central nervous system receives sensory information from many sources, and regulates the cardiovascular system at many different levels. The sympathetic nervous system provides the dominant vascular tone and participates in the control of arterial pressure by regulating arterial resistance, the release of renin, cardiac output, and venous tone.

The brain stem controls the level of sympathetic activity by integrating a number of input signals, including those from arterial baroreceptors, cardiopulmonary receptors, and from inputs within the central nervous system itself.

The sympathetic impulses that lead to vasoconstriction of the blood vessels arise from the vasomotor center in the brain stem which is the major site for integrating the excitatory and inhibitory inputs to determine the final level of activity in the sympathetic neurons. The vasomotor center is under constant modulation by fibers descending from more rostral areas of the brain, including the hypothalamus. For example, angiotensin II (ANG II) circulating in the plasma elicits indirect stimulation of the vasomotor center via an action on neurons arising from the anterior portion of the third ventricle (Hoffman and Phillips, 1976).

A major inhibitory regulator of the sympathetic nervous system is the baroreceptor reflex. With a rise in arterial pressure and the accompanying stretch of baroreceptors in the carotid sinus and aortic arch, afferent baroreceptors fibers fire more rapidly. These sensory fibers travel to the brain stem, where they synapse with interneurons located within the nucleus tractus solitarius (NTS) (Reis and Cuenod, 1965; Seller and Ilert, 1969) to inhibit sympathetic outflow. The NTS is an area of the brain stem that is a major source of inhibitory impulses which down-regulate vasoconstrictor sympathetic outflow. This provides a feedback loop in which a rise in arterial pressure stimulates the NTS, which in turn inhibits the resting level of sympathetic outflow to the resistance vessels resulting in a return to the normal resting arterial pressure.

In addition to afferents from the baroreceptors, the NTS also receives input from other neurons within the brain stem. Many of these are adrenergic and also stimulate interneurons that inhibit sympathetic tone. The transmitter from these neurons is either norepinephrine or epinephrine and acts through stimulation of alpha-receptors. Stimulation of central alpha-receptors in the NTS lowers blood pressure, in contrast to the pressor effect of stimulating alpha-receptors on the vascular smooth muscles (Zandberg et al., 1979).

Cardiopulmonary mechanoreceptors sense changes in blood volume and also may contribute to regulation of sympathetic neurotransmission through the NTS. Although these reflexes are less well understood than the arterial baroreceptors; and their central transmitters and connections less well defined, they appear to be important in the long-term regulation of blood volume. Vagal afferents from the cardiopulmonary region exert a tonic inhibition on the release of renin through connections at the NTS by reducing sympathetic outflow to the kidneys (Mancia et al., 1975).

Thus the NTS appears to be the site of integration for information from arterial baroreceptors, the cardiopulmonary receptors, and the hypothalamus concerning the central control of blood pressure. The efferent pathways influencing the cardiovascular system have been shown to be located in the spinal cord and in the brain stem. The origin of sympathetic preganglionic fibers have been well-established since 1851 (Clarke, 1851). These fibers originate from the intermediolateral cell column (IML) of the spinal cord and innervate both the heart and smooth muscle of blood vessels. Vagal preganglionic cardioinhibitory axons originate from two nuclei in the brain stem: the dorsal motor nucleus of the vagus (DMV) and the nucleus ambiguus (Nosaka et al., 1979; Ciriello and Calaresu, 1980a; Ciriello and Calaresu, 1982).

Specific anatomical connections of the baroreflex will now be discussed. The activity of the central neurons taking part in cardiovascular regulation is to a large degree determined by nervous impulses carried via primary afferent fibers of the IXth and Xth cranial nerves from the several different peripheral baroreceptor sites. The baroreceptor afferent impulses enter the NTS, a bilateral structure, via the IXth and Xth cranial nerves, where they first synapse.

Cell bodies of the primary baroreceptor neurons are located in the nodose ganglion. Specialized axon terminals of these perikarya serve as stretch receptors to detect changes in blood pressure. These baroreceptive structures are distributed in the carotid sinus, aortic arch and cardiac structures. The proximal fibers of primary baroreceptor neurons travel among the other fibers of the IXth (glossopharyngeal) and Xth (vagus) nerves and reach the brain stem approximately at the level of the obex. Some of the entering fibers take a dorsal route to the NTS but others course ventrally and follow the efferent fibers of the vagal nerve (Allen, 1923). Both dorsal and ventral afferent fibers reach the tractus solitarius and descend to the caudal levels of the brain stem. The fibers which pass through the ventral route may contain most of the primary baroreceptor afferents. Hypertension develops immediately if bilateral transections, lateral to the NTS, are made sufficiently deep to cut the ventrally located fibers. No change in blood pressure occurs if only dorsal fibers are cut (DeJong and Palkovits, 1976). The afferent fibers reach the tractus solitarius and descend through the brain stem to the most caudal portion of the tractus (Cajal, 1896).

The NTS also receives viscero-sensory projections from the Vth, VIIth, IXth and Xth cranial nerves. The cranial nerves terminating in the NTS occupy relatively distinct parts of the nucleus. The rostral part of the NTS (rostral to the level of the obex) contains cells which are mostly connected with the Vth and VIIth nerves. The IXth and Xth cranial nerves carry most of the cardiovascular information and terminate in the caudal part of the nucleus.

The NTS is divided into the medial and lateral subnuclei by the tractus solitarius itself, each having distinct cytoarchitecture and different functions. The lateral part of the NTS has been implicated as a primary respiratory center (von Euler et al., 1973). The medial part of the NTS is larger. Caudal to the obex, the right and the left NTS come closer to the midline and finally, 800-900 um behind the obex, the nucleus commissuralis is formed. On the basis of the character of the cells, the nucleus commissuralis can be considered as a mere caudal continuation of the NTS.

The caudal part of the NTS and nucleus commissuralis form the primary brain stem center for the cardiovascular reflex arc. Lesions of this area produce acute hypertension in both cats and rats (Doba and Reis, 1973; DeJong et al., 1975). Electrical stimulation of this region results in hypotension and bradycardia (Oberholzer, 1955). Similar manipulation of other portions of the NTS produce far less dramatic effects on the cardiovascular system.

The cell bodies of the baroreceptor efferent fibers have not been convincingly localized in specific nuclei of the brain stem. It has been suggested that efferent baroreceptor neurons may be located in the DMV or in the nucleus ambiguus (Gunn et al., 1968). The recent localization of cardioinhibitory neurons in these nuclei support these suggestions. The nucleus ambiguus receives direct fibers from the NTS. Dahlstrom and Fuxe (1965) provided the first description of descending monoaminergic fibers in the central control of sympathetic function. Catecholamine-containing cells of the A1 region in the ventral lateral medulla and A2 region of the NTS appear to provide monoaminergic innervation of the spinal cord, in particular the IML (Chung et al., 1975), site of the cardiovascular preganglionic sympathetic neurons. Axons leave the spinal cord and terminate in the sympathetic ganglia on the postganglionic neurons which constitute the final stage of the baroreflex arc. Henry and Calaresu (1974) have found that the cell bodies of the medullary efferents to the IML also lie in many of the reticular nuclei of the brain stem. No direct neuronal connections from the medial NTS to the spinal cord have been found. However, terminations of neuron fibers from the NTS on the various nuclei of the reticular formation suggest that baroreceptor reflexes reach the spinal cord by a multisynaptic pathway; via the NTS-reticular formation-intermediolateral nucleus of the spinal cord (Palkovits and Zaborszky, 1977).

The activity of the efferent baroreceptor neurons is influenced by higher control mechanisms originating from the cortex, hypothalamus and limbic system as well. Higher modulating centers receive information carried via primary baroreceptor afferents using an ascending multisynaptic neural pathway originating from the secondary baroreceptor neurons in the NTS. In the same manner, neuronal outputs from higher brain regions do not directly effect the peripheral sympathetic nerves but modulate activity of baroreceptor efferent nerves by connections to the NTS and spinal cord.

One of these sites of higher control originates in the paraventricular nucleus (PVN) of the hypothalamus. In addition to its well-established role in the control of posterior pituitary hormones (Hayward, 1977), the PVN has recently been implicated in the regulation of the cardiovascular system. Anatomic studies have shown that the PVN projects not only to the neurohypophysis but also to the dorsal medial medulla, particularly the NTS and the DMV (Saper et al., 1976; Sofroniew and Schrell, 1981; Swanson and Kuypers, 1980; Swanson et al., 1980; Zerihun and Harris, 1983). These investigations suggest that, on the basis of topographic separation and the morphological characteristics of retrogradely labeled PVN neurons, separate populations of neurons project to the neurohypophysis and to the dorsal medulla. Furthermore, this nucleus has been shown to play a role in hypertension as lesions of the PVN prevent and or reverse the elevation in arterial pressure due to aortic baroreceptor deafferentation or alter the development of hypertension in the spontaneously hypertensive rat (Zhang et al., 1983; Ciriello et al., 1983).

The PVN has been shown to receive direct afferent projections from the dorsal vagal complex which includes the NTS and the DMV (Sawchenko and Swanson, 1981) and these projections have been shown to relay cardiovascular afferent information (Ciriello and Calaresu, 1980c). In addition, the PVN maintains a direct reciprocal connection with the NTS and the IML. The PVN, thus, appears to form a feedback loop with the NTS to act as a possible center for modulatory control of the cardiovascular system by altering the ability of the baroreflex to regulate arterial pressure through the NTS. The descending pathways from the PVN to the NTS will now be discussed.

The physiological significance of the PVN neurons projecting to the brain stem remains to be clarified. However, electrical stimulation of the PVN has been shown to elicit an increase in arterial blood pressure and heart rate and to inhibit reflex bradycardia evoked by stimulation of the carotid sinus nerve (Ciriello and Calaresu, 1980c). Since the NTS has been shown to be the site of termination of afferent fibers from the carotid baroreceptors and the DMV has been demonstrated to contain vagal cardioinhibitory preganglionic neurons, PVN projections to these dorsal brain stem structures may participate in direct central cardiovascular regulation.

The neurochemical composition of descending PVN neurons remains in doubt. Although oxytocin and vasopressin are known primarily as circulating hormones produced by the magnocellular neurosecretory cells and released from the posterior pituitary, recent studies have revealed a substantial network of oxytocin- and vasopressin-containing fibers throughout the central nervous system. However, oxytocin and vasopressin neurons account for less than 10% of PVN neurons projecting to the brain stem (Sofroniew and Schrell, 1981). Interestingly, oxytocin- and vasopressin-stained cells account for only about 20% of the cells in the PVN that are labeled after injections of retrograde tracers into the NTS (Swanson & Sawchenko, 1980), thus suggesting that additional cell types may contribute to PVN-to-NTS modulation of sympathetic nervous activity. In fact some 30 different putative neurotransmitters have been identified either in cell bodies of the PVN or in presumed terminals (Swanson & Sawchenko, 1983). Most of these appear to be found primarily in the parvocellular division of the PVN, including ANG II. Immunohistochemical evidence that an ANG II-like peptide exists in the cell bodies of the PVN has been reported by Phillips et al. (1979) and others (Brownfield et al., 1982). Angiotensin II has also been localized in the fibers and terminals of PVN neurons by several groups (Fuxe et al., 1976; Changaris et al., 1978), suggesting that cell bodies containing ANG II could play a role in cardiovascular regulation through projections to the NTS.

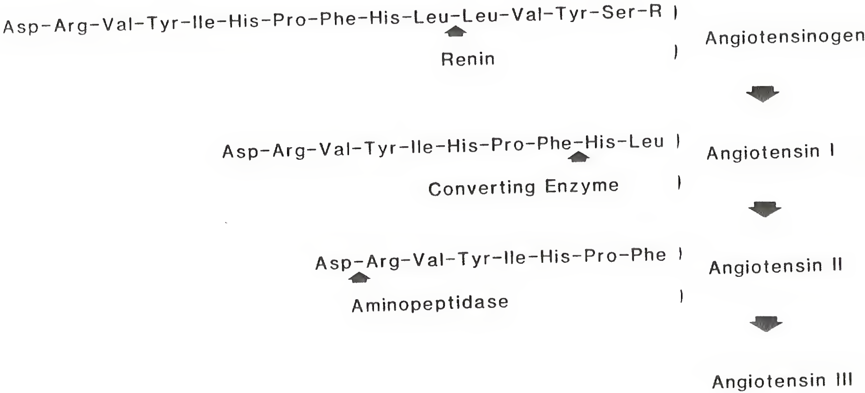
In summary, the baroreflex arc consists of a multisynaptic neuron loop. Primary neurons have their cell bodies in the nodose ganglion and connect the peripheral baroreceptor sites with the NTS. The first synapse in the baroreflex arc and also the origin of the secondary neurons are located in the caudal part of the NTS. The fibers of the secondary neurons terminate in various brain stem nuclei (dorsal vagal, ambiguus, reticular) and in higher centers which may modulate the baroreflex arc through reciprocal connections. The efferent preganglionic neurons of the arc are located in several parts of the brain stem and terminate on the cell bodies of the preganglionic sympathetic fibers in the IML.

The Brain Renin-Angiotensin System

The renin-angiotensin system was first described in terms of a renal-circulating hormone that generates ANG II in the plasma. The sequence of steps in the synthesis of circulating ANG II are shown in Figure 1. The main target organs of the active hormone are vascular smooth muscle, the adrenal gland, and the kidney. Hormonal ANG II increases blood pressure by its vasoconstrictor action, by stimulation of aldosterone secretion and subsequent sodium and water retention, and by stimulation of catecholamine release.

In the blood, angiotensin is formed by enzymatic cleavage from angiotensinogen, a glycoprotein synthesized in the liver. Angiotensinogen is split by the enzyme renin which is formed in the kidneys. As a result, angiotensinogen is often referred to as renin substrate.

Figure 1. The cascade of steps for the synthesis and degradation of angiotensin II in the peripheral circulation. R indicates the remainder of molecule.


Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Leu-Val-Tyr-Ser-R
Renin
Angiotensinogen
Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu
Converting Enzyme
Angiotensin I
Asp-Arg-Val-Tyr-Ile-His-Pro-Phe
Aminopeptidase
Angiotensin II
Angiotensin III

The action of renin leads to the generation of a decapeptide angiotensin I (ANG I) which is then cleaved to the active octapeptide, ANG II by a dipeptidyl-carbohydrolase, angiotensin converting enzyme (ACE). High concentrations of ACE are found in the endothelial lining of the pulmonary capillaries (Roth et al., 1969). Thus, the formation of plasma ANG II typifies the synthesis of peripheral hormones in that it involves several steps and several organ systems. Blood-borne ANG II produces its pressor effects in two ways; 1) direct vasoconstriction of the peripheral resistance vessels and; 2) an action on the brain to produce an increase in sympathetic outflow. The central site of action of circulating ANG II has been the subject of many investigations. The overwhelming evidence is that peripheral ANG II does not have free access to central cardiovascular control centers due to protection by the blood-brain barrier, but acts on specialized areas of the brain which lack this barrier.

The first notion of a blood-brain barrier was made by Ehrlich (1885), who observed that intravenously given dyes such as trypan blue stain all tissues of the body with the exception of the brain, spinal cord and peripheral nerves. The site of this impedance to the passage of circulating tracers was not known. Experiments with vital dyes given into the peripheral vasculature later showed that the dye was unable to pass through the endothelium of brain capillaries. The endothelial cells, basement membrane and perivascular space remained unstained (Spatz, 1934). Endothelial cells of brain capillaries, arterioles, and venules are interconnected by very close appositions of the cell membranes forming a pentalaminar structure (tight junction) (Brightman and Reese, 1969), limiting access of circulating substances into brain tissue.

As a result, penetration of ANG II into the brain after peripheral administration is poor. Penetration of radiolabeled ANG II from blood into the cerebrospinal fluid (CSF) was suggested by Johnson (1975) using autoradiography. However, the inability of endogenous plasma angiotensin to penetrate into the CSF was shown by Schelling et al. (1976). Later, Schelling et al. (1977) presented evidence that the radioactivity found in the brain by Johnson probably represented degradation products and that angiotensin does not cross the blood-brain barrier intact.

The only sites of the brain where the blood-brain barrier mechanisms are lacking are in the circumventricular organs. In circumventricular organs, such as the organum vasculosum of the lamina terminalis (OVLT), subfornical organ (SFO) and area postrema, transfer of circulating solutes from the blood to neural tissue may be of importance.

The central site of action, as far as pressor and drinking effects to plasma ANG II are concerned, has been studied in many species. In all species except the rat, the area postrema appears to mediate the central effects of circulating ANG II (Gildenberg et al., 1963; Dickinson and Yu, 1967; Joy and Lowe, 1970). Anatomic connections exist between the area postrema and the NTS and vagal nucleus. The SFO and OVLT appear to be important mediators for the central effects of peripheral ANG II in the rat (Johnson and Buggy, 1977). For instance, specific receptor neurons for circulating ANG II have been demonstrated in the SFO by Phillips and Felix (1976). Miselis et al. (1979) have demonstrated neural connections between the SFO and magnocellular neurosecretory neurons in the supraoptic and paraventricular nucleus, which represents a possible neural pathway for circulating ANG II to activate central systems known to be involved in cardiovascular control. Hartle and Brody (1984) have recently reviewed the forebrain ANG II pressor system in the rat in great detail.

In addition to being sensitive to circulating ANG II, all components of the peripheral renin-angiotensin system are present within the brain. The possibility of a separate functional renin-angiotensin system involved in the central control of blood pressure has been pursued by several groups of workers (Fischer-Ferraro et al., 1971; Yang and Neff, 1972; Day and Reid, 1976; Hoffman et al., 1976; Printz and Lewicki, 1977; Ganten et al., 1978; Hutchinson and Csicsmann, 1978; Phillips, 1980).

Angiotensinogen. The presence of angiotensinogen was first demonstrated in brain tissue of dogs (Ganten et al., 1971) and later in rabbits and sheep (Printz and Lewicki, 1977). It has also been localized in CSF of rats, dogs, sheep and man (Ganten et al., 1975a; Reid, 1977; Printz and Lewicki, 1977). Biochemical characterization of brain and plasma angiotensin indicates that they are not identical. This argues in favor of its local synthesis and against plasma contamination of the brain tissue. It appears that angiotensinogen levels in brain may be rate-limiting for ANG II formation, since injection of exogenous angiotensinogen into brain tissue has elicited drinking (Hoffman et al., 1976). This is unlike the peripheral system where the release of renin from the kidneys appears to be the rate-limiting step in the synthesis of ANG II.

Renin. Renin-like enzymes that form ANG I from angiotensinogen have been measured in brain tissue of many species including rats (Fischer-Ferraro et al., 1971; Ganten et al., 1971; Reid, 1977). There was uncertainty regarding the exact nature of this enzyme, due to the technical difficulty in the purification of renin from tissue. All renin preparations have been found to be contaminated with cathepsin D-like acid protease activity. This lead some authors to suggest identity of brain renin and cathepsin D acid protease. However, separation of acid protease activity by affinity chromatography yields differential elution of renin and protease activity (Ganten and Speck, 1978) and different optimal pH. Since this purified brain renin also cross-reacts with antibodies to renal renin, the existence of true renin in the brain is no longer a matter of contention.

The distribution of brain renin has been studied by measuring the ability to generate ANG I from renin substrate and by immunocytochemistry. Using the former, Schelling et al. (1981) have reported high concentrations of renin in the pineal and anterior pituitary glands. Other areas of the brain to contain renin include the NTS. In fact, Schelling et al. reported that the NTS renin content of spontaneously hypertensive rats was higher than normotensive controls. Antibodies to highly purified renin have been used to determine the distribution of renin by immunocytochemical techniques. Fuxe et al. (1980) reported the presence of renin-like immunoreactivity in cells of the supraoptic nucleus and PVN providing presumptive evidence that renin is present in neurons within the brain.

Angiotensin. The highest density of ANG II immunoreactivity has been found in the structures surrounding the third ventricle, the brain stem, and spinal cord. Most angiotensin immunoreactive cell bodies are found in the supraoptic nucleus and PVN (Phillips et al., 1979). There exists evidence that ANG II is costored with vasopressin in the same neurons of the PVN (Kilcoyne et al., 1981). Scattered angiotensin immunoreactive nerve cell bodies have also been observed in the perifornical area and in the medial amygdaloid nucleus. It seems possible that most of the angiotensin immunoreactive nerve terminals demonstrated in various parts of the brain and spinal cord belong to the cells of the PVN since most of the supraoptic nerve cells only project to the posterior pituitary gland (Swanson and Sawchenko, 1983). The distribution of angiotensin immunoreactive nerve terminals ranges from high density in the median eminence, substantia gelatinosa, and sympathetic lateral column to low density in the striatum, and several structures in the brain stem including the locus coeruleus and NTS (Ganten et al., 1978).

Quantitative measurement of angiotensin levels in the brain has proven difficult due to the high brain angiotensinase activity (Ganten et al., 1975b). The first reports on the presence of angiotensin determined by radioimmunoassay in brain tissue by Fischer-Ferraro et al. (1971) and Ganten et al. (1971) indicated that mostly ANG I but also ANG II were present. ANG II concentrations in the picogram to nanogram range per gram brain tissue have been reported (Ganten et al., 1978; Phillips, 1984); however local concentrations in specific areas may be much higher.

To determine whether the ANG II measured in the brain by radioimmunoassay is identical to the peripheral peptide, the technique of high-pressure liquid chromatography has recently been employed. The results demonstrate that the ANG II extracted from rat brain comigrates with peripheral ANG II with the largest concentration of ANG II in the hypothalamus (Lang et al. 1983; Phillips, 1984). The second largest concentration of ANG I and ANG II is found in the brain stem, suggesting that ANG II may play a functional role at the level of the NTS.

Angiotensin Converting Enzyme. ACE is a hydrolase enzyme which cleaves histidyl-leucine from the carboxyl end of the decapeptide ANG I to form ANG II. Enzyme concentrations in brain are lower than in lung but significantly higher than in plasma and other tissues (Roth et al., 1969; Yang and Neff, 1972; Chevillard and Saavedra, 1982). There is a small but measurable amount of converting enzyme in CSF. The distribution of ACE in brain tissue is variable, with very high concentrations in the choroid plexus, SFO, and area postrema. Relatively high levels of ACE can be found in the brain stem (Yang and Neff, 1972) and recent studies by Chevillard and Saavedra (1982) report significant amounts within the NTS.

Angiotensin Receptors. Angiotensin II receptors, as defined by saturable and specific binding sites, have been found in the brains of various species of mammals (Bennett and Snyder, 1976; Sirett et al., 1977; Harding et al., 1981; Mendelsohn et al., 1984). In rats, the highest density of ANG II receptors are found in the PVN of the hypothalamus, NTS and area postrema. Relatively high concentrations are also reported in the suprachiasmatic nucleus of the hypothalamus, olfactory tracts and the inferior olivary nuclei. Receptors for ANG II are highly concentrated in relatively few sites in the central nervous system suggesting that ANG II may perform a modulatory role in a restricted number of precise functions within the brain.

Central Sites of Action. Central administration of ANG II or renin produces an increase in water intake (Fitzsimons, 1971) and blood pressure and increased secretion of vasopressin and adrenocorticotrophic hormone (Buckley, 1972; Severs and Daniels-Severs, 1973; Reid and Ramsay, 1975). Except under unusual circumstances, circulating renin and ANG II do not penetrate parts of the brain outside the circumventricular organs. Currently, two areas in the rat brain and one area in the dog brain have been accepted as receptor sites for plasma- or CSF-borne ANG II. In the rat brain, the SFO appears to be a site of action for blood-borne angiotensin to exert its central effects. The OVLT appears to have the dual ability to detect both blood-borne ANG II and intraventricular ANG II (Phillips et al., 1977a)

The other ANG II receptor site is the area postrema which is of great importance in the dog (Ferrario and Barnes, 1981) but of no known importance in the rat (Haywood et al., 1980). In addition to these circumventricular organs, there are areas of cardiovascular regulation with concentrated binding sites for ANG II not accessible to either blood- or CSF-borne ANG II. This suggests that the brain renin-angiotensin system may play an important role at these sites in central regulation of autonomic function.

In summary, there are generally four lines of evidence which support the view that local formation of ANG II occurs by an independent brain renin-angiotensin system: 1) the biochemical identification of key proteins needed to synthesize ANG II in brain tissue and CSF; 2) the localization of specific ANG II binding sites in various parts of the central nervous system involved in cardiovascular regulation; 3) the histological visualization of neuronal fibers and cells with ANG II-like immunoreactivity; and 4) the effects of intraventricular injection of renin and ANG II upon the arterial pressure and the sympathetic and endocrine functions of normal and hypertensive animals.

Significance

As we have seen, the NTS is the primary site of central cardiovascular regulation; and therefore a likely candidate for neuropeptide modulation. Although the cardiovascular effects of intravenous and intraventricular administration of ANG II have been well investigated, it is not known whether a system of ANG II-containing neurons has direct action at the NTS. The evidence suggesting that the brain renin-angiotensin system may be important on the NTS include:

1) The colocalization of ANG II with vasopressin in paraventricular neurons of the hypothalamus.

2) Projections of paraventricular neurons to various sites in the brain stem, including the NTS.

3) Evidence of the components for local synthesis of ANG II within the NTS including:

    a) renin

    b) angiotensin converting enzyme

    c) ANG I

    d) ANG II

4) The high concentration of specific angiotensin receptors within the NTS.

Within this dissertation, evidence will be presented directed at helping to define the role of angiotensin in the NTS. Microinjection of ANG II directly into the NTS will be performed to elucidate any biological activity at this specific site. Additionally, data will be presented to determine the effects of angiotensin on the function of the baroreflex arc at the NTS.

CHAPTER 2
CARDIOVASCULAR ACTIONS OF MICROINJECTIONS OF ANGIOTENSIN II IN THE BRAIN STEM OF RATS

Introduction

Recent evidence indicates that the hypothalamus gives rise to a large number of peptide-containing fibers that may be associated with blood pressure regulation. The paraventricular and supraoptic nuclei (SON) of the hypothalamus contain cells that synthesize and secrete vasopressin and oxytocin into the blood stream from the posterior pituitary. In addition, neuroanatomic techniques have implicated caudal connections of the PVN in the central control of cardiovascular regulation (Swanson and Sawchenko, 1983). For example, the PVN supplies vasopressin-containing fibers to the NTS in the brain stem (Swanson, 1977; Zimmerman, 1981).

Since anatomic and electrophysiologic studies have now firmly established the NTS as the site where primary baroreceptor afferents first synapse (Reis and Cuenod, 1965; Seller and Ilert, 1969), there has been a new interest in the role of peptides in the NTS. Matsuguchi et al. (1982) have shown that microinjection of vasopressin directly into the region of the NTS elicits tachycardia and a pressor response. These results suggest that vasopressin acts in the region of the NTS to exert a central action on the circulation.

Angiotensin II is also a peptide that has central effects on circulation. When injected into the ventricular system, ANG II produces a potent pressor response (Severs et al., 1967; Phillips, 1980; Phillips et al., 1982b). There is immunohistochemical evidence that an ANG II-like peptide exists in the cell bodies of the PVN and SON (Phillips et al., 1979). Several structures in the brain stem implicated in cardiovascular regulation have been shown to possess ANG II receptors or immunoreactive fibers, including the area postrema (AP) and NTS (Sirett et al., 1977; Ganten et al., 1978; Phillips et al., 1979; Weyhenmeyer and Phillips, 1982). This, in addition to the presence of fibers from the PVN and SON projecting to the brain stem, has led to this investigation into the effects of direct microinjection of ANG II into the NTS.

The AP is a circumventricular organ that has been shown to have a major role in the pressor response of blood-borne ANG II in many species (Gildenberg et al., 1963; Dickinson and Yu, 1967; Joy and Lowe, 1970). Due to its close anatomic proximity to the NTS and the demonstration that the AP is part of a medullary neural pathway that augments central sympathetic outflow (Ciriello and Calaresu, 1980b), studies were performed to investigate whether an interaction exists between the AP and NTS in the central effects of ANG II on circulation.

Methods

Male Sprague-Dawley rats (250-350 g) were used in all experiments. Rats were housed in individual cages in a temperature controlled room on a 12-h light-12-h dark cycle. All experiments were performed under urethan anesthesia (1.2-1.4 g/kg, ip). The femoral artery was catheterized with PE-50 tubing and connected via a Statham P23Gb transducer to a Gould chart recorder for continuous measurement of blood pressure and heart rate. A similar catheter was placed in the femoral vein for peripheral administration of test drugs. The animals were then placed in a stereotaxic frame with the head flexed downward to an angle of 45 degrees. After a midline incision through the skin, the dorsal neck muscles were retracted to expose the foramen magnum. The caudal portion of the fourth ventricle was exposed by incising the atlantooccipital membrane, and when necessary, retracting the cerebellum with a spatula. Microinjections of ANG II (Sigma, St. Louis, MO.) were made via a glass micropipette (60-80 micron outer tip diameter) connected by PE-10 tubing to a remote Hamilton microliter syringe (no. 701N) driven by a Sage infusion pump (model 341). The vehicle for all injections was artificial cerebrospinal fluid (CSF) (Myers, 1971), except where noted. The pH of all solutions injected into the region of the NTS was between 7.0 and 8.0 with a volume of 0.1 microliter (ul) given over 30 s. Each rat received only two injections into each NTS, one injection of the peptide, and one injection of the vehicle in a random order. The procedures were then duplicated in the contralateral NTS, with injections 0.5 mm rostral to the caudal tip of the area postrema, 0.5 mm lateral to the midline, and 0.9 mm below the dorsal neural surface (Zandberg and DeJong, 1977).

Effect of the vehicle. Thirteen rats were tested for blood pressure and heart rate responses to injection of 0.9% saline or artificial CSF vehicle alone into the NTS. The same volume of the other vehicle was administered 30-60 min after injection of either 0.9% saline or artificial CSF, when blood pressure and heart rate had returned to baseline. The tests were repeated in the region of the contralateral NTS.

Dose response. Eighteen rats were tested for blood pressure and heart rate responses to three doses of ANG II injected into both the right and the left NTS. Doses tested were 50, 250, and 500 ng dissolved in 0.1 ul artificial CSF. Tests of the middle dose (250 ng) were repeated with injection directly into the AP, as well as intravenously.

Intravenous injections were dissolved in a final volume of 0.5 ml artificial CSF.

Area postrema ablation. Six rats received a thermal ablation of the AP. Under binocular-microscopic guidance, the region was first blotted with a cotton-tipped applicator. A thermal lesion was then made by touching an electrocautery copper wire (0.75 mm diam) momentarily to the exposed tissue of the AP. After a return to stable baseline blood pressure and heart rate, injections of 250 ng ANG II were administered into the AP or NTS. Blood pressure and heart rate responses were recorded and compared with injections before ablation.

Peripheral angiotensin blockade. Six rats received intravenous administration of 10 ug saralasin (Sigma), an ANG II competitive inhibitor in 0.5 ml saline. Intravenous ANG II (250 ng in 0.5 ml saline) was administered 3 min later to test the efficacy of ANG II blockade. After a return to baseline, the intravenous dose of saralasin was repeated. Injections of ANG II (250 ng in 0.1 ul CSF) were performed into either the right or left NTS 3 min later to test the possibility that any hemodynamic responses could be due to leakage into the periphery.

Verification of injection sites and statistics. The injection sites were marked at the end of each experiment by injecting 0.1 ul of Evans blue dye at stereotaxic coordinates identical to those used in the drug injections

(Palkovits et al., 1974). Thirty minutes after dye injection, the rats were perfused transcardially with 50 ml 0.9% saline followed by 50 ml of 10% buffered formaldehyde, the brains were removed, and 40 um frozen sections of the brain stem cut and alternate sections Luxol fast blue-cresyl violet stained for histological verification of injection sites as well as the extent of AP ablation in the appropriate animals.

Changes in mean arterial pressure (MAP) and heart rate were measured 1 min after completion of injection. Statistical comparisons between the groups were performed using Student's t test. A P value of less than 0.05 was considered significant. All reported values are mean ± SE.

Results

Effect of the vehicle. The effect of NTS injections of 0.9% saline and artificial CSF is shown in Figure 2. The mean resting blood pressure and heart rate values for all rats tested were 90.0 ± 3.6 mmHg and 378 ± 13 beats/min, respectively. There were no significant changes after injections of artificial CSF (90.6 ± 3.6 mmHg and 377 ± 13 beats/min). Heart rate and blood pressure consistently and significantly decreased after equal-volume injections of 0.9% saline (76.6 ± 5 mmHg and 353 ± 11 beats/min).

Figure 2. Comparison of heart rate (HR, beats/min) and mean arterial pressure (MAP, mmHg) change after microinjection of 2 vehicles, 0.9% saline and artificial cerebrospinal fluid (CSF) into the nucleus tractus solitarius. Numbers in parentheses indicate sample size. Values are means ± SE. Significantly different from baseline: (*P < 0.05, Student's t test).

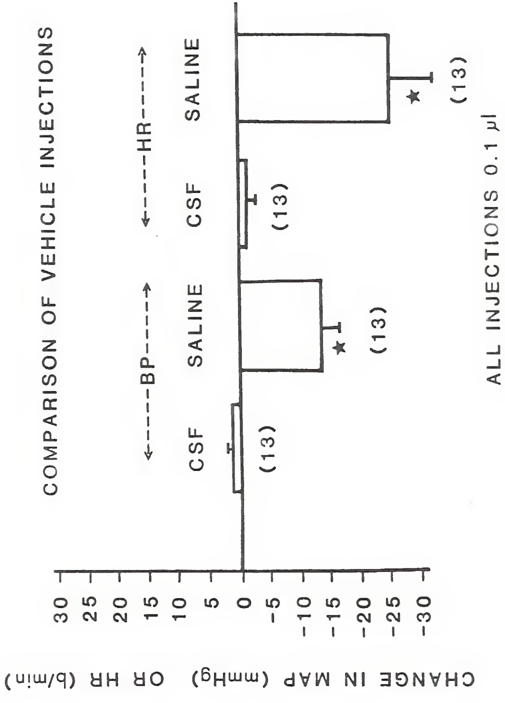
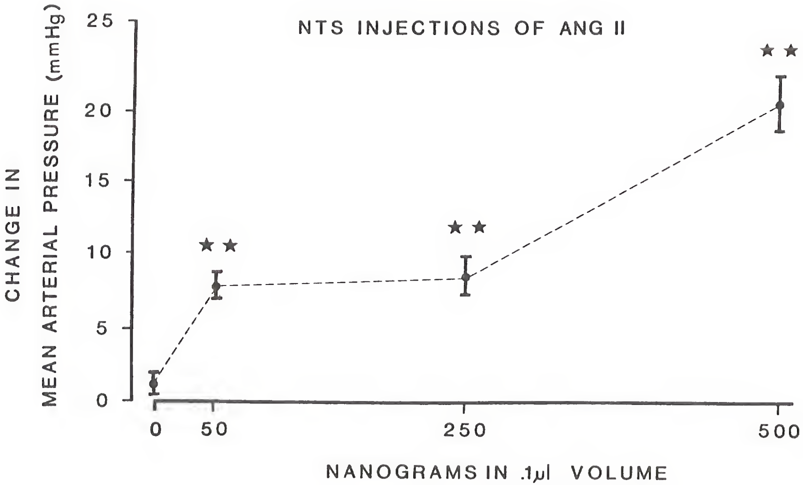

COMPARISON OF VEHICLE INJECTIONS
←-----BP-----→
←-----HR-----→
CSF
SALINE
CSF
SALINE
(13)
(13)
(13)
(13)
★
★
30
25
20
15
10
5
0
-5
-10
-15
-20
-25
-30
CHANGE IN MAP (mmHg) OR HR (b/min)
ALL INJECTIONS 0.1 µl

Figure 3. Changes in mean arterial pressure (MAP) after microinjection of three doses of angiotensin II (ANG II) into nucleus tractus solitarius. Vertical bars, SE; $n = 6$ samples/dose. Significance: $*P < 0.01$, Student's t test compared with injection of artificial cerebrospinal fluid vehicle alone.


NTS INJECTIONS OF ANG II
CHANGE IN
MEAN ARTERIAL PRESSURE (mmHg)
25
20
15
10
5
0
★ ★
★ ★
★ ★
0
50
250
500
NANOGRAMS IN .1µl VOLUME

Dose response. Unilateral injection of ANG II in the CSF vehicle into the right or left NTS was associated with a significant increase ($P < 0.01$) in blood pressure at all doses, as shown in Figure 3. Values for the region of the right and left NTS were not significantly different and were therefore combined into a single treatment group at each dose. The largest dose (500 ng) produced the greatest change (20.5 ± 2.3 mmHg) in arterial pressure, whereas the smaller doses (250 and 50 ng) produced smaller changes (8.6 ± 0.6 and 7.8 ± 1.4 mmHg, respectively). Heart rate was consistently but insignificantly increased after injection at all doses, which is in contrast to significant decreases seen after either AP or intravenous administration. Figure 4 illustrates a comparison of heart rate response to 250 ng ANG II given under these various conditions. Significant decreases are seen only with intravenous (-45 ± 10 beats/min, $P < 0.01$) and AP (-14 ± 6 beats/min, $P < 0.05$) injections.

Area postrema ablation. The blood pressure and heart rate responses to 250 ng of ANG II injected into the NTS were not significantly altered by thermal ablation of the AP. Preablation injections into the NTS resulted in a rise in MAP of 8.6 ± 0.6 mmHg. After thermocautery of the AP, 250 ng of ANG II microinjected into the NTS resulted in a pressor response of 11.6 ± 1.4 mmHg (Figures 5 and 6). Direct injection of 250 ng ANG II into the AP yielded a

Figure 4. Effect on heart rate (HR, beats/min) of different injections of angiotensin II (250 ng). Numbers in parentheses indicate sample size; values are means ± SE. AP, area postrema, SAR, saralasin. Significantly different from injection into nucleus tractus solitarius (NTS) alone $*P < 0.05$, Student's t test, $** P < 0.01$.

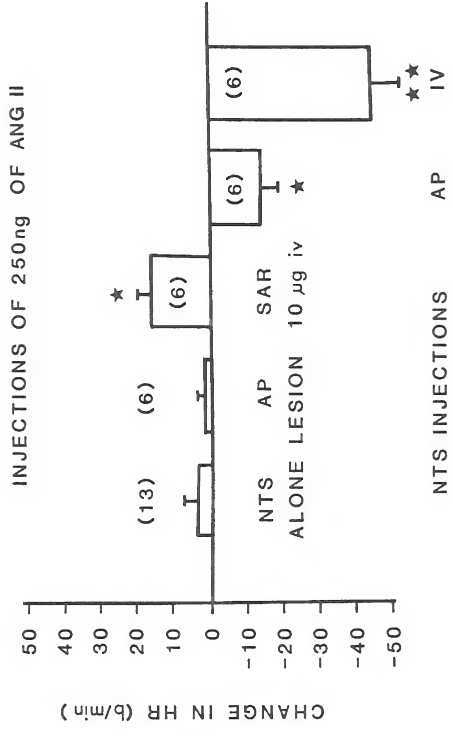
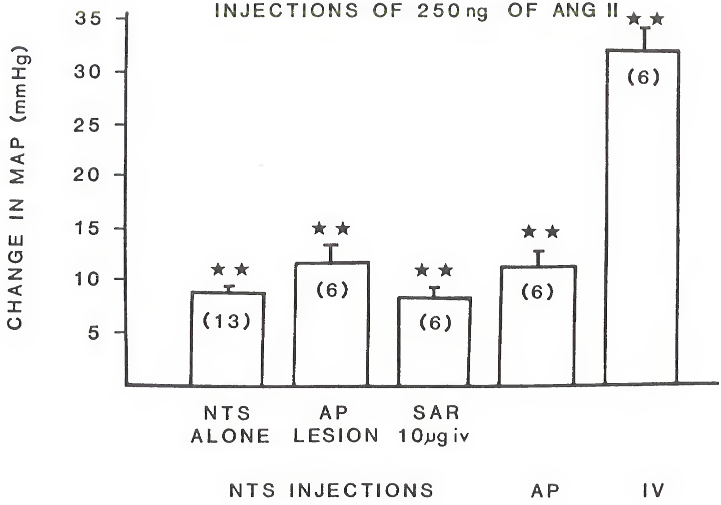

INJECTIONS OF 250ng OF ANG II
CHANGE IN HR (b/min)
50
40
30
20
10
0
-10
-20
-30
-40
-50
(13)
(6)
(6)
(6)
(6)
NTS ALONE
AP LESION
SAR 10 µg iv
AP
IV
NTS INJECTIONS
★
★
★★

Figure 5. Effect on mean arterial pressure (MAP) of different injections of angiotensin II (250 ng). Numbers in parentheses indicate sample size; values are means ± SE. See Figure 3 for abbreviations. Significantly different from artificial cerebrospinal fluid injection into NTS: **$P < 0.01$, Student's t test).


INJECTIONS OF 250ng OF ANG II
CHANGE IN MAP (mmHg)
35
30
25
20
15
10
5
★★
(13)
★★
(6)
★★
(6)
★★
(6)
★★
(6)
NTS ALONE
AP LESION
SAR 10μg iv
NTS INJECTIONS
AP
IV

significant ($P < 0.01$) rise in MAP (11.3 ± 6.0 mmHg). After thermal ablation of the AP, equal-dose injections of ANG II into the AP resulted in no change in either blood pressure (0.3 ± 0.7 mmHg) nor heart rate (0.7 ± 0.2 beats/min).

Peripheral angiotensin blockade. After peripheral ANG II-receptor blockade, ANG II microinjections (250 ng) into the NTS produced significant increases in both blood pressure (8.3 ± 1.2 mmHg) and heart rate (15 ± 4.0 beats/min). When compared with preblockade responses there was no significant difference in pressor response to microinjection of 250 ng of ANG II (Figure 5). Heart rate, which was not significantly altered by ANG II microinjection before peripheral ANG II blockade, was significantly elevated after saralasin pretreatment (15 ± 4.0 beats/min, Figure 4).

Histology. Microscopic examination of the brain stem revealed that the dye microinjected into the NTS had spread to an average diameter of 2.5 ± 1.0 mm rostrocaudally, 1.6 ± 0.9 mm mediolaterally, and 0.5 ± 0.3 mm dorsoventrally, apparently following the structure of the NTS. The dye-stained area illustrated in Figure 7 included the NTS in all cases and occasionally included parts of the dorsal motor nucleus of the vagus and the AP. In each rat receiving an AP ablation, all of the AP was ablated without apparent damage to the NTS.

Figure 6. Lack of attenuation of pressor response to nucleus tractus solitarius (NTS) microinjection of 250 ng angiotensin II (ANG II) after either peripheral saralasin (SAR) pretreatment or area postrema (AP) ablation. A:pulsatile pressor response to microinjection of ANG II into the NTS alone. Arrow, injection time. B:response to ANG II microinjected into NTS (second arrow) 3 min after peripheral ANG II blockade with 10 ug intravenous saralasin (first arrow). C:response to ANG II microinjected into NTS 30 min after thermal ablation of AP.

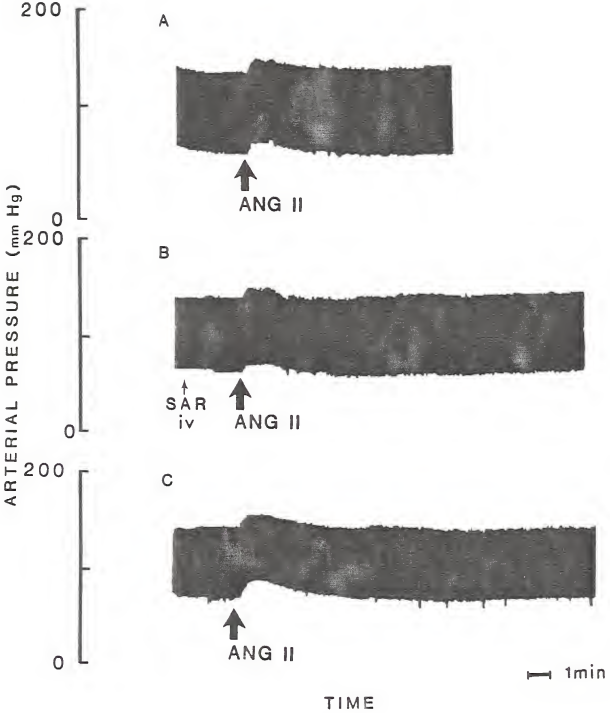
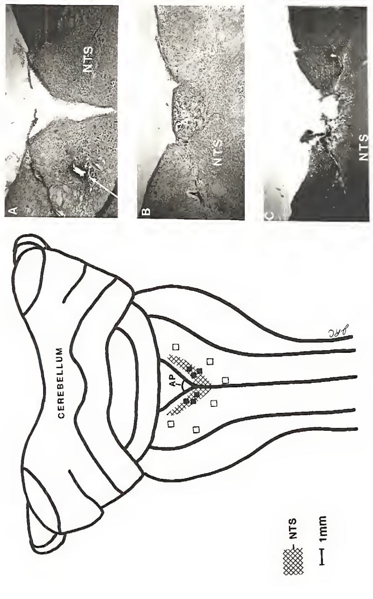

ARTERIAL PRESSURE (mm Hg)
200
0
A
ANG II
200
0
B
SAR
iv
ANG II
200
0
C
ANG II
1min
TIME

Figure 7. Diagrammatic representation (left) showing dorsal aspect of medulla and caudal cerebellum. Solid squares, approximate location of injection sites producing pressor responses to 500 ng of angiotensin II (ANG II). Open squares, sites of injection yielding no response. These sites were distributed approximately 1 mm away from nucleus tractus solitarius (NTS) coordinates. Right, representative Luxol fast blue-cresyl violet-stained coronal sections of brain stem. A (6.5 mm posterior to interauricular line); injection site (arrow) of 0.1 ul dye at stereotaxic coordinated identical to those used for injection of ANG II into NTS of rat. B (7.0 mm posterior): close proximity of NTS and intact area postrema (AP). C (7.0 mm posterior); NTS intact after thermal ablation of AP.


A
NTS
B
NTS
C
NTS
CEREBELLUM
AP
NTS
1mm

Discussion

The results clearly indicate that direct injection of ANG II into the NTS produces a consistent, significant, dose-related pressor response over a range of 50-500 ng ANG II. This effect is not due to spread of the injected ANG II to the AP. Removal of the AP by thermal ablation did not alter the response to NTS injections, as shown in Figure 6. The doses of ANG II used in this study to produce increases in blood pressure are larger than those required when ANG II is introduced into the cerebral ventricles (Severs et al., 1967; Phillips, 1980; Phillips et al., 1982b). This may be due to the fact that the pressor effect following intraventricular administration of ANG II consists of two distinctive components. One involves the release of vasopressin from the posterior pituitary by activation of cells in the paraventricular and supraoptic hypothalamus. Intraventricular ANG II also raises arterial pressure by increasing vasoconstrictor activity through central components of the sympathetic nervous system. At this point, it is not clear whether one or both of these systems represent the mechanism responsible for the increase in blood pressure by angiotensin in the NTS. Finally, it is not appropriate to compare the dose used in this study with those used in intraventricular studies, since Phillips et al. (1977a) using cream plugs to isolate parts of the ventricular system, have shown that the site of action of intraventricular ANG II lies in the anterior third ventricle, whereas this study examined the effects of ANG II in the brain stem. The 250 ng dose of ANG II was chosen in the comparative studies for two reasons: 1) it was the smallest dose that produced consistent responses from both the NTS and AP, and 2) for the experiment with saralasin, a larger dose would require an increased amount of the ANG II antagonist. Unfortunately at such doses, saralasin has agonistic properties that would render the test ambiguous. At 10 ug iv, however, saralasin has full antagonistic action against ANG II, and therefore these were the doses of choice. Direct injection of ANG II into the AP did not produce a pressor response which was significantly different from injections into the NTS alone. The change in heart rate after AP injection was opposite that with NTS providing evidence against the effects of NTS injection being due to spread of the injectate and subsequent AP stimulation. ANG II injected into the NTS consistently produced a slight but not significant increase in heart rate. Injections of ANG II into the area postrema, however, consistently produced a significant decrease in heart rate.

To control for the possibility that the central ANG II injection might be leaking into the systemic circulation, saralasin was injected intravenously to block peripheral effects of ANG II. The pressor response was not altered after peripheral ANG II blockade (Figure 6). Under this condition, injection of ANG II into the NTS caused an even greater elevation of heart rate than NTS injections alone. This implies that an interaction may exist between peripheral ANG II and central ANG II having an effect on the baroreceptor reflex. In view of the role of the AP as an ANG II receptor site, which has been established in dogs (Barnes and Ferrario, 1981), the AP may serve as a receptor mediator for blood-borne ANG II to act on the NTS. In our studies, AP lesions had no effect on the cardiovascular responses to injections into the region of the NTS alone. Unlike some species, Haywood et al. (1980) have shown that the AP does not mediate the pressor effect following peripheral or intraventricular administration of ANG II in the rat. All these data suggest that the NTS contains active sites for the action of ANG II, and the effects are not due to spread of ANG II to the AP. The results parallel those of Matsuguchi et al. (1982), who have shown elevation of blood pressure with injections of vasopressin in the NTS. In their study, injection sites very close to but not within the NTS did not produce the pressor response, implying that only one area was sensitive to the injection and that spread of the injectate from those sites was not a confounding variable. The location of effective injection sites in the present study was only in the NTS and not in adjacent sites. This study and that of Matsuguchi et al. demonstrate specific actions of peptides localized within the region of the NTS.

An interesting observation was the importance of the vehicle. In the study comparing artificial CSF with saline as a vehicle, it was found that CSF produced no change in blood pressure or heart rate, whereas saline (0.9%) caused a significant depressor response and a significant decrease in heart rate. Future studies of peptide actions in the brain stem should therefore be carried out only with artificial CSF, not with saline as the vehicle. The differences in the effect of the vehicles may be due to the lack of calcium ions when 0.9% saline is used as a vehicle. Calcium is required for neurotransmitter release and neural activation (Douglas, 1968). The artificial CSF contains calcium ions, which are not present in the saline vehicle. When saline is injected, it may dilute available calcium to below effective levels, thereby inactivating neurons in the region.

The results are in keeping with a hypothesis that there are ANG-containing terminals in the region of the NTS which play a role in the control of circulation. These responses need to be investigated further to test whether they are dependent or independent of vasopressin-induced responses. Since ANG II-like immunoreactivity has been shown in the PVN cells (Phillips et al., 1979), it seems possible that fibers projecting from the PVN to the NTS include ANG II-containing processes. The data in this study offer physiological support for this prediction, although immunohistochemical demonstration of such a connection remains to be made. The fact that the NTS does contain ANG II-like immunoreactive fibers has been recently confirmed by others (Brownfield et al., 1982), but the source of these fibers is not known. Lesioning studies and stimulation studies of PVN also have demonstrated pressor effects that can be abolished by cutting fiber pathways between the PVN and the NTS (Phillips et al. 1982b). These studies will be consistent with the notion that, in addition to vasopressin-containing fibers, there are also angiotensin-containing fibers emanating from the peptide-containing neurons of PVN. A peptide projection from the PVN to the NTS is a possible route by which the hypothalamus could modulate the brain stem control of circulation. The influence from the baroreceptor reflex was unexpectedly small, if present at all. Although only a minor increase in heart rate was seen with injection with ANG II into the NTS, these small increases were consistent with each animal tested and were exaggerated to a significant change when saralasin was used to inhibit peripheral ANG II. Thus, it would appear that endogenous brain ANG II released into the NTS could interact with peripheral levels of ANG II to contribute to the net output of the baroreceptor reflex.

CHAPTER 3
MECHANISM OF PRESSOR EFFECTS BY ANGIOTENSIN IN THE NUCLEUS TRACTUS SOLITARIUS OF RATS

Introduction

The presence of receptors for ANG II (Mendelsohn et al., 1984) and ANG II immunoreactivity in the NTS (Ganten et al., 1978; Phillips et al., 1979; Weyhenmeyer and Phillips, 1982), an area known to be involved in cardiovascular regulation, led to the previous study on the effects of exogenously applied ANG II in that area. Microinjection of ANG II into the NTS of urethan-anesthetized rats yields a significant dose-dependent elevation in blood pressure and consistent but not significant increases in heart rate similar in onset and duration to those resulting from intraventricular administration. The effect was not due to either leakage of the peptide into the peripheral circulation or spread of the injectate to the nearby area postrema, an area with known cardiovascular responsiveness to ANG II (Ferrario et al., 1970; Ferrario et al., 1972).

Angiotensin has central pressor action, and the pressor effect following intraventricular administration has been well investigated (Buckley, 1972; Severs et al., 1970;

Severs and Daniels-Severs, 1973). A portion of the pressor response to centrally administered angiotensin is mediated through the hypothalamus, resulting in the release of vasopressin from the posterior pituitary (Severs et al., 1970; Buckley, 1972; Keil et al., 1975; Phillips, 1980; Phillips et al., 1982b). For example, intraventricular ANG II produces a smaller pressor response in rats of the Brattleboro strain, which are homozygous for diabetes insipidus, than it does in Long-Evans controls (Hutchinson et al., 1976). Additional evidence that a hormonal mechanism is partially responsible for the pressor effect of ANG II lies in the fact that central injection of angiotensin in hypophysectomized rats also produces a significantly reduced (less than 50% of controls) pressor effect compared with intact animals (Severs et al., 1970). Since Brattleboro rats do not produce the hormone vasopressin and rats that have been surgically hypophysectomized exhibit impaired vasopressin secretion, the data suggest that the central blood pressure response to ANG II in the conscious rat is in part due to vasopressin release.

There is evidence that increased sympathetic tone contributes the remainder of the central blood pressure response to ANG II (Severs et al., 1967; Severs et al., 1970; Buckley, 1972). Recordings from sympathetic efferent nerves show increased activity following central activation by angiotensin (Aars and Akre, 1968; Kiem and Sigg, 1971). In the definitive study by Severs et al. (1970), interruption of vasopressin secretion by surgical hypophysectomy, and subsequent ganglionic blockade of the sympathetic nervous system effectively abolished the pressor response to centrally administered ANG II. Thus the central pressor effect of angiotensin involves both humoral and neural mechanisms in conscious animals. Hypophysectomy effectively abolishes the former and ganglionic blockade the latter.

In the present study, an attempt is made to investigate the mechanism by which ANG II microinjected into the NTS elicits a pressor response in urethan-anesthetized rats. A comparison can then be made between the mechanisms of the NTS pressor response and that seen with the intraventricular route of administration in conscious rats. To determine the contribution of neural versus humoral mechanisms, three protocols were employed. One group of rats was surgically hypophysectomized to impair vasopressin release from the pituitary prior to ANG II injection into the NTS. In another group of animals the vascular effects of vasopressin were abolished by using a vasopressin vascular antagonist before injection of ANG II into the NTS. The results were compared with those from intact controls to determine the contribution of pituitary hormonal pressor agents, specifically vasopressin, in the response. Secondly, intact rats were treated with the short-acting ganglionic blocker, hexamethonium chloride, to eliminate the sympathetic nervous system as a source of pressor activity before ANG II injections into the NTS. Using these protocols, each of the two pathways known to contribute to the pressor response seen with intraventricular injections of ANG II was selectively eliminated and any contribution to the pressor response following ANG II microinjection into the NTS could be evaluated.

Another member of the angiotensin family, angiotensin I (ANG I) was tested for cardiovascular effects in the NTS for the following reasons. During recent years, there have been reports that ANG I and II are present in CSF and brain tissue. However, there are considerable discrepancies in the distribution and concentration. Preliminary work has indicated that ANG I and II can be found in high levels in the brain stem (Fischer-Ferraro et al., 1971; Changaris et al., 1977; Hutchinson and Csicsmann, 1978).

Fitzsimons (1971) observed that ANG I, when administered centrally, was almost as potent as ANG II in causing rats to drink. He therefore suggested that angiotensin-converting enzyme (ACE) in the brain could act to convert ANG I, synthesized within the brain, to ANG II. Initial reports by Roth et al. (1969) that ACE could be found in the brain in relatively high concentrations have led to investigation of the levels within local regions of the brain. Reports followed that ACE could be found in high levels in the medulla (Yang and Neff, 1972), and, in recent studies, Chevillard and Saavedra (1982) have reported that high concentrations of ACE could be found in the NTS.

However, it has not been established whether ANG I exists in the same fibers and structures in the brain stem as ANG II. To test whether ANG I would be converted to ANG II and have the same effects as ANG II, ANG I has been applied directly to the NTS.

Methods

Male Sprague-Dawley rats (250-350 g) were used in all experiments. Rats were housed in individual cages in a temperature-controlled room on a 12-h light-12-h dark cycle. All experiments were performed under urethan anesthesia (1.2-1.4 g/kg, ip). The femoral artery was catheterized with PE-50 tubing and connected via a Statham P23Gb transducer to a Gould chart recorder for continuous measurement of blood pressure and heart rate. A similar catheter was placed into the femoral vein when appropriate for peripheral administration of test drugs.

To expose the medulla, the animals were placed in a stereotaxic frame with the head flexed downward to an angle of 45 degrees. After a midline incision through the skin, the dorsal neck muscles were retracted with sutures exposing the foramen magnum. The caudal portion of the fourth ventricle was exposed by incising the atlantooccipital membrane and, when necessary, gently retracting the cerebellum with a spatula. Microinjections of peptides were performed via a glass micropipette (60-80 micron outer tip diameter) connected by PE-10 tubing to a remote Hamilton microliter syringe (no. 701N) driven by a Sage infusion pump (model 341). The vehicle for all injections was artificial CSF (Myers, 1971). The pH of all solutions injected into the NTS was between 7.0 and 8.0 with a volume of 0.1 ul given over 30 s. At least 30 min were allowed before injections into the NTS were repeated. Each rat received injections only into the right NTS; the coordinates 0.5 mm rostral to the caudal tip of the area postrema, 0.5 mm lateral to the midline, and 0.9 mm below the dorsal neural surface were used (Zandberg and DeJong, 1977).

Comparison of response to ANG I with that of ANG II. Six rats were tested for blood pressure and heart rate responses to microinjection of three doses each of ANG I and II. The doses used for ANG II were 50, 250, and 500 ng, whereas 250, 500, and 1,000 ng were used for ANG I. Artificial CSF was used as a control injection, and all injections of the peptides were given in random order in a volume of 0.1 ul. A dose-response curve was constructed for each of the peptides.

Effect of sympathetic blockade on ANG II response in NTS. The blood pressure and heart rate responses to 500 ng of ANG II injected into the NTS were tested in six rats that had been given a single injection of the ganglionic blocker, hexamethonium chloride. A control injection of 0.1 ul of CSF was given into the NTS, and at least 30 min were allowed before additional tests were carried out. Thirty minutes after a test dose of ANG II (500 ng) into the NTS, hexamethonium chloride (20 mg/kg) was given intravenously. Intravenous injections were given with a final volume of 0.5 ml with normal saline as the vehicle. Three minutes after administration of the short-acting ganglionic blocker, a second injection of ANG II (500 ng) was given, and the blood pressure and heart rate responses were recorded. Thirty minutes after ganglionic blockade, when the blood pressure had returned to baseline, a third injection of ANG II (500 ng) was given to test whether the cardiovascular responsiveness of the NTS to ANG II had returned to normal. Thirty minutes later a second control injection of artificial CSF was given. The cardiovascular responses 3 and 30 min after hexamethonium treatment were compared with preblockade values to determine whether ganglionic blockade affected the onset, magnitude, or duration of the effect of ANG II microinjection into the NTS.

ANG II in the NTS of hypophysectomized rats. Eight hypophysectomized male Sprague-Dawley rats were obtained from Charles River Breeding Laboratory (Wilmington, MA.). After recovering in our laboratory for three days, daily water consumption was measured to provide a measurement of the efficiency of the surgical hypophysectomy, since rats lacking vasopressin are known to be polydipsic. Rats consuming less than 20 ml/100 g/day were eliminated from the study. After acclimatization for at least 1 and no more than 2 weeks after hypophysectomy, the rats were prepared as described previously. Each rat received two injections of artificial CSF (0.1 ul) and two injections of ANG II (500 ng) into the NTS in a random order. The blood pressure and heart rate responses were compared with those after equal-dose injections into intact Sprague-Dawley rats.

Blockade of vascular vasopressin receptors. Six rats were prepared as previously described. Each rat was tested with injections of artificial CSF (0.1 ul) and ANG II (500 ng) into the NTS. After a return to stable blood pressure and heart rate, each animal received a test dose of Arg^8-vasopressin (10 ng, iv) dissolved in 0.5 ml normal saline. Sixty minutes later, the vasopressin vascular antagonist, $d(CH_2)_5Tyr(Me)$-arginine vasopressin (1 ug, iv) was given. A postblockade injection of Arg^8-vasopressin (10 ng, iv) was given 15 min later to test the efficacy of vasopressin blockade. Thirty minutes later, ANG II (500 ng) was microinjected into the NTS, and blood pressure and heart rate responses were monitored.

Thirty minutes after ANG II administration, the postblockade injection of Arg^8-vasopressin was repeated to ensure that vasopressin-receptor blockade was present during administration of ANG II.

Verification of injection sites and hypophysectomy. The injection sites were marked at the end of each experiment by injecting 0.1 ul of Evans blue dye at stereotaxic coordinates identical to those used for drug injections (Palkovits et al., 1974). Thirty minutes after dye injection, the rats were perfused transcardially with 50 ml normal saline followed by 50 ml of 10% buffered formaldehyde, the brain was removed, and 40 micron coronal sections of the brain stem were cut and Luxol fast blue-cresyl violet stained for histological verification of the injection site. In hypophysectomized rats, coronal sections of the midbrain were also cut to visually assess the degree of pituitary destruction.

Measurements and statistics. Pulsatile arterial pressure and heart rate were recorded throughout the procedures. MAP was calculated from the pulsatile record. Changes in MAP and heart rate were measured 1 min after completion of injections. Statistical comparison between the groups was performed using Student's t test. Multiple comparison of means was accomplished by the use of ANOVA followed by the Newman-Keuls test; $P < 0.05$ was considered significant. All reported values are means ± SE.

Results

Comparison of response to ANG I with that of ANG II. The dose-response curves for the pressor responses to ANG I and II are shown in Figure 8. Six animals were tested with the following doses of each peptide in addition to CSF controls. ANG I (250 ng) produced a significant ($P < 0.05$) elevation in MAP from 87.2 ± 2.2 to 90.7 ± 1.8 mmHg, a 500 ng dose produced increases ($P < 0.001$) from 86.2 ± 3.4 to 91.0 ± 3.3 mmHg, and the highest dose (1,000 ng) yielded a significant ($P < 0.001$) rise from 87.9 ± 4.6 to 97.9 ± 4.0 mmHg. The CSF vehicle produced a nonsignificant change in MAP from 86.2 ± 4.4 to 86.8 ± 3.6 mmHg. Although no significant difference could be found between the pressor response to 250 and 500 ng of ANG I, the response to 1,000 ng was significantly ($P < 0.001$) greater than the lower doses. In no instance was the heart rate significantly changed after injection of ANG I into the NTS. The three doses of ANG II used were 50, 250, and 500 ng. The smallest dose (50 ng) produced the smallest increase in MAP from 85.4 ± 4.0 to 91.3 ± 4.7 mmHg, whereas 250 ng ANG II produced a pressor response from 85.9 ± 1.3 to 94.5 ± 0.9 mmHg. The largest dose (500 ng) produced a rise in MAP from 86.2 ± 4.4 to 101.5 ± 3.5 mmHg. All doses of ANG II produced a significant ($P < 0.001$) pressor response compared with CSF controls, but only the largest dose produced a response significantly ($P < 0.001$) larger than the smaller doses. The heart rate response to ANG II injections was significant ($P < 0.05$) only for the largest dose (500 ng), which produced a rise from 274 ± 31 to 291 ± 28 beats/min. The smaller doses produced nonsignificant increases from 271 ± 21 to 282 ± 21 beats/min for 50 ng ANG II and from 269 ± 21 to 273 ± 11 beats/min for 250 ng ANG II. Although the smaller doses did not produce significant increases in heart rate, the response was one of consistent but slight increases.

Effect of sympathetic blockade on ANG II response in NTS. Preblockade test doses of artificial CSF and ANG II (500 ng) in six rats produced blood pressure increases of 0.6 ± 3.2 and 15.5 ± 1.6 mmHg, respectively. A heart rate response to CSF was not apparent, yielding a change of -1 ± 2 beats/min. A significant ($P < 0.05$) increase in heart rate was associated with ANG II injection from 274 ± 31 to 291 ± 28 beats/min. Treatment with the ganglionic blocker, hexamethonium chloride, alone caused a highly significant ($P < 0.001$) depression of blood pressure and heart rate from 69.3 ± 5.8 mmHg and 317 ± 26 beats/min to 41.8 ± 4.0 mmHg and 255 ± 22 beats/min. However, by 3 min after blockade when the second injection of ANG II was given, the blood pressure and heart rate had returned to 75% of normal. A 3-min postblockade injection of ANG II into the NTS did not produce any changes in heart rate, 277 ± 4 beats/min before injection of ANG II to 275 ± 5 beats/min after. However, small but significant ($P < 0.05$) increases in blood pressure were seen with ANG II 3 min after ganglionic blockade, 65.5 ± 3.7 vs. 70.2 ± 3.2 mmHg, indicating that the ganglionic blockade was either no longer complete or a very slight hormonal component was present in the response.

Thirty minutes after hexamethonium treatment, injections of 500 ng of ANG II into the NTS produced a significant ($P < 0.001$) pressor response from 62.4 ± 4.6 to 77.4 ± 4.2 mmHg and a nonsignificant increase in heart rate from 297 ± 8 to 304 ± 9 beats/min. The response to ANG II 3 and 30 minutes after ganglionic blockade were compared by ANOVA to preblockade responses. The pressor responses are shown in Figure 9. A significant reduction ($P < 0.001$) in the response occurs at 3 min after blockade. At 30 min after blockade there was no significant difference in the pressor response compared to preblockade values. There did not appear to be any difference in either the onset or duration of the pressor response after recovery from ganglionic blockade as shown in Figure 10. The heart rate response to injections of ANG II into the NTS in these animals was highly variable during blockade. The only incidence where significant changes in heart rate occurred

Figure 8. Comparison of mean arterial pressure changes after microinjection of 2 peptides, angiotensin I (ANG I) and angiotensin II (ANG II), into nucleus tractus solitarius. Vehicle in each case was artificial cerebrospinal fluid. Sample size is 6 for each peptide at each dose. Values are means ± SE. Significant difference from vehicle injections: *$P < 0.05$; **$P < 0.001$.

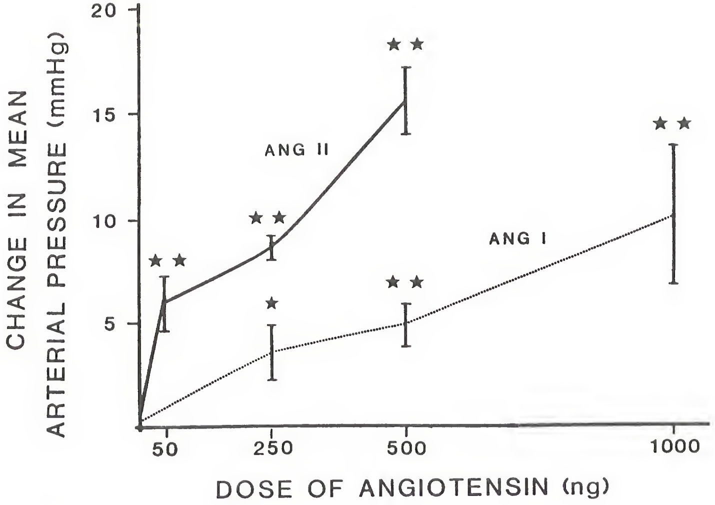
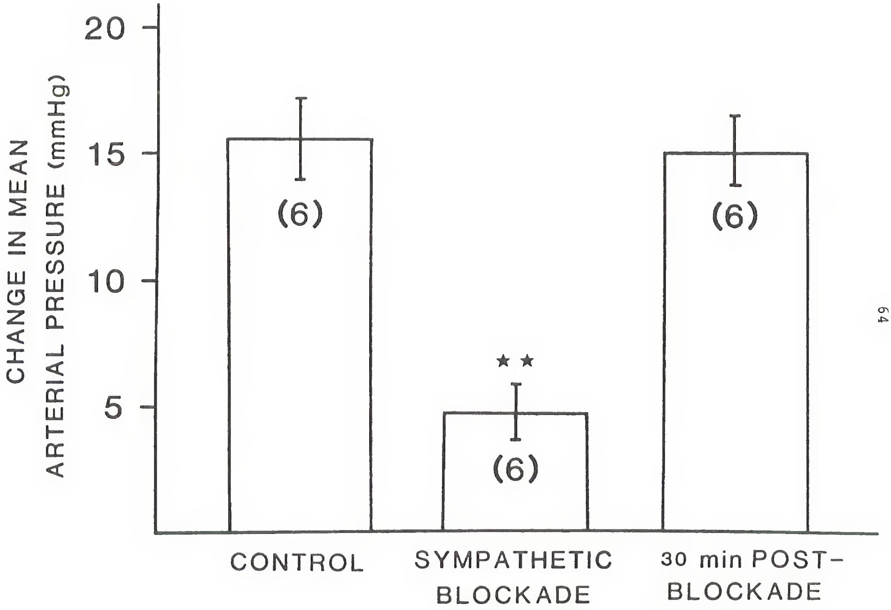

CHANGE IN MEAN ARTERIAL PRESSURE (mmHg)
20
15
10
5
ANG II
ANG I
★ ★
★
50
250
500
1000
DOSE OF ANGIOTENSIN (ng)

Figure 9. Mean arterial pressure change as a result of injection of 500 ng angiotensin II (ANG II) into nucleus tractus solitarius after ganglionic blockade with hexamethonium (20 mg.kg, iv.). Control, blockade, and 30 min postblockade results are shown. Numbers in parentheses indicate sample size. Values are means ± SE. Significant difference from control injections of ANG II: **$P < 0.001$.


CHANGE IN MEAN
ARTERIAL PRESSURE (mmHg)
20
15
10
5
(6)
★★
(6)
(6)
CONTROL
SYMPATHETIC BLOCKADE
30 min POST-BLOCKADE

in response to injections of ANG II were in the preblockade injections where an increase ($P < 0.05$) was noted.

ANG II in the NTS of hypophysectomized rats. A comparison of the responses in hypophysectomized and intact rats is shown in Table 3-1. The daily water consumption of intact Sprague-Dawley rats averaged 12.6 ± 1.1 ml/100 g body weight, whereas hypophysectomized animals of the same strain consumed 23.8 ± 1.9 ml/100 g. An unpaired t test showed a highly significant ($P < 0.001$) polydipsia in the hypophysectomized rats compared with intact controls. Injections of artificial CSF into the hypophysectomized rats did not produce any changes in MAP or heart rate. ANG II (500 ng) injections into the NTS of hypophysectomized rats produced inconsistent changes in heart rate. Blood pressure, however, was significantly ($P < 0.001$) elevated in both groups. In the intact rats, 500 ng ANG II significantly ($P < 0.001$) increased MAP from 86.2 ± 4.4 to 101.5 ± 3.7 mmHg and in hypophysectomized animals increased blood pressure from 68.7 ± 3.6 to 83.5 ± 4.4 mmHg.

Blockade of vascular vasopressin receptors. A preblockade injection of artificial CSF produced no change in either blood pressure (1.2 ± 0.5 mmHg) or heart rate (-4 ± 5 beats/min). Preblockade microinjection of ANG II (500 ng) yielded a highly significant ($P < 0.001$) elevation in blood pressure from 82.7 ± 4.1 to 96.8 ± 2.8 mmHg and no change in heart rate (335 ± 11 vs. 338 ± 11 beats/min).

Intravenous administration of the vasopressin antagonist, d$(CH_2)_5$Tyr(Me) AVP (1 ug in 0.5 ml saline), did not produce any detectable changes in either heart rate or blood pressure. Fifteen minutes after vasopressin blockade, intravenous administration of Arg^8-vasopressin (10 ng) produced no change in blood pressure (from 86.3 ± 4.9 to 90.2 ± 4.7 mmHg) or heart rate (from 368 ± 13 to 360 ± 14 beats/min). Injection of 500 ng Ang II into the NTS yielded a pressor response of 15.7 ± 1.7 mmHg but did not change heart rate (357 ± 14 vs. 360 ± 17 beats/min). Seventy-five minutes after vasopressin blockade and 30 min after ANG II microinjection, intravenous Arg^8-vasopressin (10 ng) still had no effect on blood pressure (84.8 ± 3.7 vs. 88.8 ± 3.2 mmHg) or heart rate (364 ± 13 vs. 360 ± 13 beats/min).

Comparison of the pressor responses with microinjection of ANG II into the NTS showed that there was no significant difference in the magnitude of the pressor response to 500 ng in the hypophysectomized animals (14.8 ± 3.2 mmHg), after the vasopressin vascular antagonist (15.7 ± 1.7 mmHg), or in intact Sprague-Dawley controls (15.5 ± 1.6 mmHg). Figure 11 summarizes the CSF and ANG II pressor responses in the three groups of animals.

Histology. Microscopic examination of the brain stem revealed that the spread of dye microinjected into the NTS apparently followed the structure of the NTS. This confirms

TABLE 3-1. Daily water consumption and cardiovascular parameters for intact vs. hypophysectomized rats receiving injections into the NTS.

	Intact	Hypophysectomized
Body wt, g	287.0 ± 8.4	259.4 ± 5.2
Daily H_2O consumption, ml/100 g	12.6 ± 1.1	23.8 ± 1.9
MAP, mmHg		
Baseline	86.2 ± 4.4	68.7 ± 3.6
CSF	86.8 ± 3.6	70.1 ± 6.3
ANG II (500 ng)	101.5 ± 3.7*	83.5 ± 4.4*
HR, beats/min		
Baseline	273 ± 31	260 ± 17
CSF	269 ± 26	259 ± 14
ANG II (500 ng)	291 ± 28	270 ± 14

Values are means ± SE for 6 rats/group. NTS, nucleus tractus solitarius; MAP, mean arterial pressure; HR, heart rate; CSF, cerebrospinal fluid; ANG II, angiotensin II. *P< 0.001 compared with appropriate controls; H_2O consumption in hypophysectomized animals compared with that of intact controls; ANG II responses compared with CSF vehicle responses.

Figure 10. Comparison of pressor response to angiotensin I (ANG I) and angiotensin II (ANG II) and comparison of ANG II response before, during, and 30 min after sympathetic blockade with hexamethonium (HEX). Arrows, time of injection.

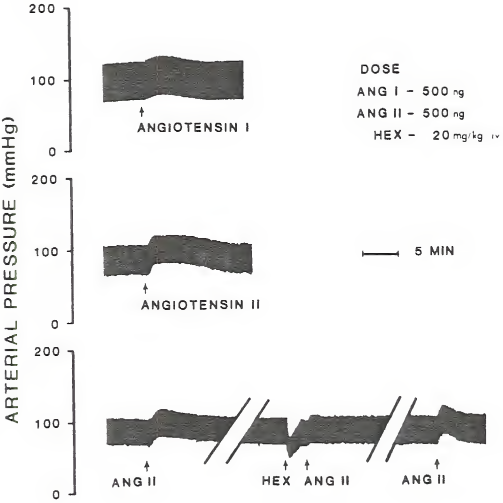
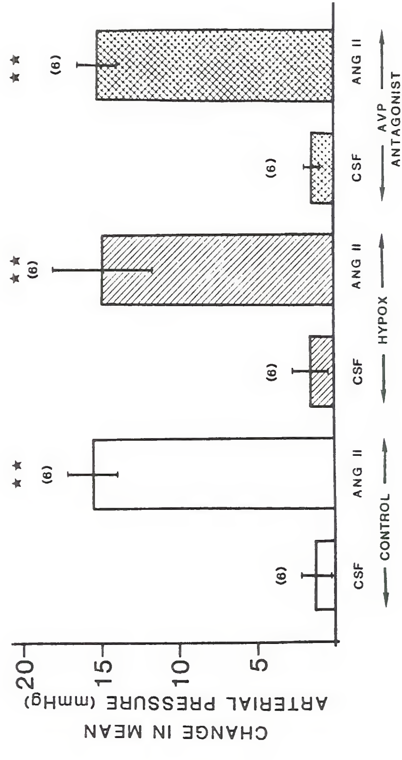

ARTERIAL PRESSURE (mmHg)
200
100
0
ANGIOTENSIN I
200
100
0
ANGIOTENSIN II
200
100
0
ANG II
HEX
ANG II
ANG II
DOSE
ANG I - 500 ng
ANG II - 500 ng
HEX - 20 mg/kg iv
5 MIN

Figure 11. Effect on mean arterial pressure of injections of artificial cerebrospinal fluid (CSF) and angiotensin (ANG II, 500 ng) into nucleus tractus solitarius of intact and hypophysectomized (hypox) rats and after vascular blockade of vasopressin receptors with 1 ug iv. d$(CH_2)_5$Tyr(Me) arginine vasopressin (AVP).[5] Numbers in parentheses indicate sample size. Values are means ± SE. Significant difference from appropriate artificial CSF control: $^{**}P < 0.001$. No significant difference in magnitude of pressor response to ANG II between 3 groups.


CHANGE IN MEAN ARTERIAL PRESSURE (mmHg)
20
15
10
5
(6)
(6)
★★
(6)
(6)
★★
(6)
(6)
★★
CSF
ANG II
CSF
ANG II
CSF
ANG II
CONTROL
HYPOX
AVP ANTAGONIST

the distribution reported in the previous study using the same procedure. The dye-stained area included the NTS in all cases and rarely included parts of the dorsal motor nucleus of the vagus and the area postrema. Histological verification of surgically hypophysectomized animals indicated, upon completion of the study, complete destruction of both the neuro- and adenohypophysis in all operated subjects. There was no evidence of pituitary tissue in either the sella turcica or coronal sections of the midbrain.

Discussion

The initial studies in this series presented a physiological function associated with ANG II in an area of the brain stem not accessible to blood-borne angiotensin by showing pressor responses to microinjection of ANG II into the NTS. This was the first evidence for a functional role of central ANG II in the brain stem. In this study, the mechanism by which ANG II causes an increase in blood pressure in the NTS was investigated.

The pressor response apparently arises largely from an increase in sympathetic outflow. The results of the studies with hypophysectomized rats and with vasopressin and sympathetic blockade are compatible with this hypothesis. Hypophysectomy or antagonism of the pressor properties of vasopressin did not reduce the pressor effect of ANG II in the NTS, whereas ganglionic blockade with hexamethonium chloride nearly abolished any pressor activity. The observation that administration of the vasopressin vascular antagonist alone did not produce any decrease in blood pressure indicates that circulating vasopressin is not a major contributor to MAP in the urethan-anesthetized rat. Thus the use of urethan as an anesthetic is not a confounding variable that might have masked the release of vasopressin in response to microinjection of ANG II into the NTS. These results indicate that the pituitary does not contribute appreciably to the pressor response seen with injections of ANG II into the NTS. This is different from the pressor response to intraventricular injections of ANG II in conscious rats that have been demonstrated to involve a major contribution from pituitary release of vasopressin as well as sympathetic activation (Severs et al., 1970; Buckley, 1972; Severs and Daniels-Severs, 1973; Keil et al., 1975; Phillips, 1980; Phillips et al., 1982b).

The ganglionic blocking agent hexamethonium was chosen for several reasons. 1) Hexamethonium is a relatively short-acting blocker, and, in contrast to nicotine and related compounds, it does not block transmission in autonomic ganglia by producing a prolonged depolarization in the membrane potential of ganglion cells. 2) It is a quaternary ammonium salt and thus is confined primarily to the extracellular space. Therefore penetration of the blood-brain barrier is limited, insuring that it's blockade of the response to ANG II in the NTS is not due to an effect on the neurons within the midbrain, medulla, or sympathetic lateral column. This class of blocking agents inhibits conduction in the sympathetic and parasympathetic nervous system. Since the dominant tone in the peripheral vasculature is sympathetic, blockade of the autonomic nervous system results in vasodilation and a fall in blood pressure.

As a result the basal arterial pressure for ANG II injections during blockade was significantly ($P < 0.001$) lower than preblockade levels. The pressor activity of humoral vasoactive compounds is inversely related to the basal blood pressure (Wilder, 1962). Had vasopressin been a component of the response, it would have been expected to cause a significant elevation in blood pressure during ganglionic blockade. In fact, Severs et al. (1970) found that after hexamethonium blockade, in intact conscious rats, intraventricular ANG II produced a significantly larger pressor response than controls. This was explained by the same vasopressin release acting on an initial hypotension produced by the blocker. A very slight pressor response to ANG II was found after hexamethonium treatment in the present study. This could be due to one of several factors. Conceivably, there could be a slight vasopressin component in the response seen only in the hypotensive states. This would not contradict the lack of difference in pressor activity of ANG II between intact and hypophysectomized animals. The results in hypophysectomized animals and animals in which the vascular actions of vasopressin are blocked indicated that vasopressin was not an active pressor agent. Therefore sympathetic activation must be considered to be mainly responsible for the pressor effect of ANG II on the NTS.

The results of the study comparing ANG I with ANG II indicate that ANG I also has pressor activity when applied directly in the NTS. In the dose range tested, ANG I was much less potent than ANG II. These studies do not discriminate between the possibility that ANG I itself has some pressor activity or whether the ANG I is converted to the octapeptide ANG II within the NTS. When conversion of ANG I to ANG II is prevented by the presence of converting enzyme inhibitors, ANG I has no pressor activity when administered either centrally or peripherally (Unger et al., 1982). These data, in addition to the presence of ACE within the NTS, allow us to conclude that the administration of ANG I into the NTS leads to local formation of ANG II, which in turn produces an increase in blood pressure through central activation of the sympathetic nervous system.

Administration of ANG II into the cerebral ventricles produces significant increases in heart rate and blood pressure. In these studies, injection of ANG II into the NTS led to very small increases in the heart rate, which were consistent in all animals tested. Only at the highest dose (500 ng) did heart rate rise significantly. Elevation of heart rate by central mechanisms can occur by two pathways: 1) activation of sympathetic outflow directly to the cardiac tissue or 2) inhibition of the parasympathetic tone. There is evidence that intraventricular administration of ANG II increases heart rate not through sympathetic activation but via inhibition of cardiac vagal tone (Lee et al., 1980). These results indicate that the pressor effect of ANG II at the level of the NTS occurs through central activation of the sympathetic nervous system but is apparently not effective in the inhibition of cardiac parasympathetic nerves seen after intraventricular administration.

When compared with intraventricular administration, the doses of ANG II used in this study to produce increases in blood pressure may at first seem large (Severs and Daniels-Severs, 1973; Phillips et al., 1982b). However, it is felt that the pressor response of 500 ng is reasonable for three reasons: 1) with intraventricular administration, central inhibition of parasympathetic tone to the heart increases cardiac output, which contributes to an increase in blood pressure (Lee et al., 1980), not seen with injections into the NTS; 2) approximately one-half of the pressor response to intraventricular ANG II occurs secondary to vasopressin release in the conscious rat (Severs et al., 1970), also not seen with injections into the NTS; and 3) intraventricular injection of 500 ng ANG II in conscious hypophysectomized rats by Severs et al. (1970) led to an increase in MAP of 17 mmHg, which is not significantly different from the magnitude of the response in the NTS in which the same dose was used. It is concluded that the efferent system activated by ANG II in the NTS descends from the medulla but does not have ascending components to the hypothalamus that result in the release of vasopressin. The source of the endogenous ANG II at the NTS may be local or delivered from the paraventricular nucleus of the hypothalamus, since cell bodies that contain immunoreactive ANG II have been found in both regions (Ganten et al., 1978; Phillips et al., 1979).

In summary, these studies show that microinjection of ANG I and II into the NTS produces an immediate increase in arterial pressure in the urethan-anesthetized animal. This pressor response appears to be mediated largely through activation of descending sympathetic fibers and is not affected by surgical hypophysectomy or blockade of the vasoconstrictor effects of vasopressin. Immunohistochemical evidence of a high-density network of ANG II-positive nerve terminals within the brain stem and sympathetic lateral column (Ganten et al., 1978) imply that there may exist central systems that use ANG II specifically to control activity in the sympathetic nervous system. In light of the immunohistochemical evidence, the present physiological findings support the view that brain ANG II and ANG II-related peptides may be important in the central mechanisms of blood pressure control. In addition to effects via actions in the hypothalamus, there is evidence for a pressor action of ANG II mediated by the NTS.

CHAPTER 4
NEUROPEPTIDE EFFECTS IN THE NTS: ANGIOTENSIN SPECIFICITY AND ITS ROLE IN HYPERTENSIVE RATS

Introduction

The NTS, first synapse of baroreceptor afferents as they enter the brain stem (Seller and Ilert, 1969; Palkovits and Zaborszky, 1977), exerts tonic inhibition over the sympathetic nervous system. For example, bilateral lesions of these nuclei produce fulminating hypertension in the rat (Doba and Reis, 1973) and chronic hypertension in other species (Nathan and Reis, 1977; Ferrario and Barnes, 1981). Conversely, electrical stimulation or chemical stimulation of the cells of the NTS with the excitatory amino acid, L-glutamate results in an acute depressor response (Talman et al., 1980).

Recent neuroanatomic and electrophysiologic studies have implicated brain stem projections of hypothalamic nuclei in the central control of sympathetic function through modulation of the NTS (Swanson and Sawchenko, 1983). Peptide-containing fibers project from the PVN and SON in the hypothalamus to the NTS (Buijs, 1978; Zimmerman, 1981). The presence of receptors for ANG II (Mendelsohn et al.,

1984) and ANG II immunoreactivity in the NTS (Ganten et al., 1978; Phillips et al., 1982a) has led to a series of investigations concerning the cardiovascular effects of exogenously applying ANG II directly to the NTS. Microinjection of ANG II (50-500 pmole) into the NTS increases mean arterial pressure (MAP) in urethan-anesthetized rats through activation of the sympathetic nervous system, and thus represents a possible pathway for hypothalamic modulation of the sympathetic nervous system.

Intraventricular administration of many peptides produces a pressor response including ANG II (Severs et al., 1970; Buckley, 1972), substance P (Fuxe et al., 1979), bradykinin (Lambert and Lang, 1970), vasoactive intestinal peptide (VIP) (Casto and Phillips, 1983), and vasopressin (Pittman et al., 1982). Substance P (Cuello and Kanazawa, 1978), VIP (Sims et al., 1980), somatostatin (Fuxe et al., 1979), oxytocin and vasopressin (Buijs, 1978) have been localized within the NTS by immunocytochemical methods. For these reasons, it was decided to investigate whether these peptides possess similar cardiovascular functions within the NTS by direct injection.

To date only two neuropeptides have been demonstrated to consistently produce an increase in MAP when injected into the NTS, angiotensin and vasopressin (Matsuguchi et al., 1982). It was therefore necessary to investigate whether one of these two peptides mediated the common response by testing whether saralasin would have any effect on the vasopressin pressor effect, and whether a vasopressin antagonist would alter the ANG II pressor effect.

Numerous central peptidergic mechanisms for the development and maintenance of hypertension have been proposed in the spontaneously hypertensive rat (SHR). One possible cause of maintained hypertension in these rats is an increase amount of ANG II in the brain. Phillips et al. (1982a) have demonstrated an increased distribution of ANG II immunoreactivity in the NTS of SHR. The responsiveness of the NTS to ANG II was examined in these rats and their Wistar-Kyoto (WKY) controls.

Methods

Male Sprague-Dawley (SD) rats (250-300 g) were used in all experiments, except where noted. SHR and WKY rats were obtained from Charles River Breeding Laboratory (Wilmington, MA). Rats were housed in individual cages in a temperature-controlled room on a 12-h light-12-h dark cycle. All experiments were performed under urethan anesthesia (1.2-1.4 g/kg, ip). The femoral artery was catheterized with PE-50 tubing and connected via a Statham P23Gb transducer to a Gould chart recorder for continuous measurement of blood pressure and heart rate. The femoral vein was similarly catheterized. The trachea was cannulated with curved tubing so as not to obstruct the airway when the head was flexed.

To expose the medulla, the animals were placed in a stereotaxic frame with the head flexed downward to an angle of 45 degrees. After a midline incision through the skin, the dorsal neck muscles were retracted with sutures exposing the foramen magnum. The caudal portion of the fourth ventricle was exposed by incising the atlantooccipital membrane. Microinjections of all peptides (Sigma, St. Louis, MO.) were performed via double-barrel glass micropipettes (60-80 um outer tip diameter) connected by PE-10 tubing to remote Hamilton microliter syringes (no. 701N) driven by two Sage infusion pumps (model 341). When possible one barrel of the micropipette was filled with dye to mark the site of injection. The vehicle for all injections was artificial cerebrospinal fluid (CSF). The pH of all solutions injected into the NTS was between 7.0 and 8.0 with a volume of less than or equal to 100 nl given over 30 s. At least 60 min were allowed before injections into the NTS were repeated. Each rat received injections only into the right medial NTS; the coordinates 0.5 mm rostral to the caudal tip of the area postrema, 0.5 mm lateral to the midline, and 0.5 mm below the dorsal surface of the neural tissue were used (Zandberg et al., 1979).

Dose response. Sprague-Dawley rats were tested for blood pressure and heart rate responses to six doses of ANG II and the artificial CSF vehicle. The doses of ANG II used were 0.1, 1, 10, 50, 250, and 500 pmoles. Each subject received only two doses of ANG II and the vehicle. The order of administration was randomized in each animal.

Cardiovascular actions of neuropeptides localized in NTS. Six peptides which have been localized within the NTS were tested for cardiovascular responses. A selected dose of 500 pmole, which corresponds to the largest dose of ANG II tested, was used. Six groups of animals received three separate injections of one of the peptides, and one of the artificial CSF vehicle.

Blockade of angiotensin and vasopressin receptors in NTS. One side of a double-barrel micropipette was back-filled with a solution containing 10 nmole/ul saralasin. The other side was back-filled with a solution containing 1 nmole/ul ANG II. The micropipette was lowered into the right medial NTS. Six rats received an injection of 100 pmole ANG II. After a return to resting MAP and HR, the NTS was pretreated with 1 nmole saralasin. Three minutes later, 100 pmole ANG II was microinjected. Sixty minutes later the ANG II injection was repeated. Blood pressure and heart rate responses were recorded and compared before, during and after blockade of angiotensin receptors in the NTS. To further test specificity and demonstrate independence of the vasopressin and ANG II pressor effect, one barrel of a micropipette was back-filled with the long-lasting vasopressin antagonist $d(CH_2)_5Tyr(Me)$ arginine vasopressin. This antagonist shows no agonistic properties either peripherally or in the NTS and has been shown to completely block any pressor activity of vasopressin in the NTS (Matsuguchi et al., 1982). The remaining barrel was back-filled with a solution containing ANG II. Three doses of ANG II were microinjected in a random order into the NTS and the cardiovascular responses recorded. Sixty minutes after the final dose, 1 ug of the vasopressin antagonist was injected into the NTS. Thirty minutes later, the three doses of ANG II were repeated as before. As a final procedure the micropipette was removed and a single barrel micropipette filled with Arg-vasopressin positioned at the exact coordinates of the prior micropipette. A dose of 100 pmole Arg-vasopressin was microinjected into the NTS to test for complete blockade of vasopressin receptors during the ANG II microinjections. In a third group of animals the procedure was repeated using the angiotensin antagonist, saralasin and vasopressin, to investigate the role of angiotensin receptors in the vasopressin pressor response. In this group of animals only one dose of vasopressin (10 pmole) was used due to rapid tachyphylaxis to repeated injection of vasopressin.

Pressor response to ANG II in the SHR. Six SHR and six WKY rats were tested with four doses of ANG II which have been shown to produce pressor responses in Sprague-Dawley rats. The doses tested were 50, 100, 250, and 500 pmoles and the artificial CSF vehicle. The order of testing was randomized in each animal. Blood pressure and heart rate responses in the three groups of animals were recorded and compared. In a separate group of animals, the blood pressure effect to a peripheral vasoconstrictor was tested. Graded doses of phenylephrine (1-12 ug, iv) were administered as bolus injections (volume < 50 ul) through the venous catheters of urethan-anesthetized rats of the S-D and SHR strain to assess the responsiveness to peripheral vasoactive agents.

Verification of injection sites and statistics. The injection sites were marked at the end of each experiment by injecting 100 nl of Evans blue dye. Thirty minutes after dye injection, the rats were perfused transcardially with 50 ml normal saline followed by 50 ml of 10% buffered formaldehyde, the brain was removed, and 40 micron coronal sections of the brain stem were cut and Luxol fast blue-cresyl violet stained for histological verification of injection site.

Pulsatile arterial pressure and heart rate were recorded throughout the procedures. MAP was calculated from the pulsatile record. Change in mean arterial pressure and heart rate were measured 1 min after completion of injections. Statistical comparison between the groups was performed using Student's t test. Multiple comparison of means was accomplished by the use of analysis of variance (ANOVA) followed by the Newman-Keuls test when appropriate. $P < 0.05$ was considered significant. All reported values are means ± SE.

Results

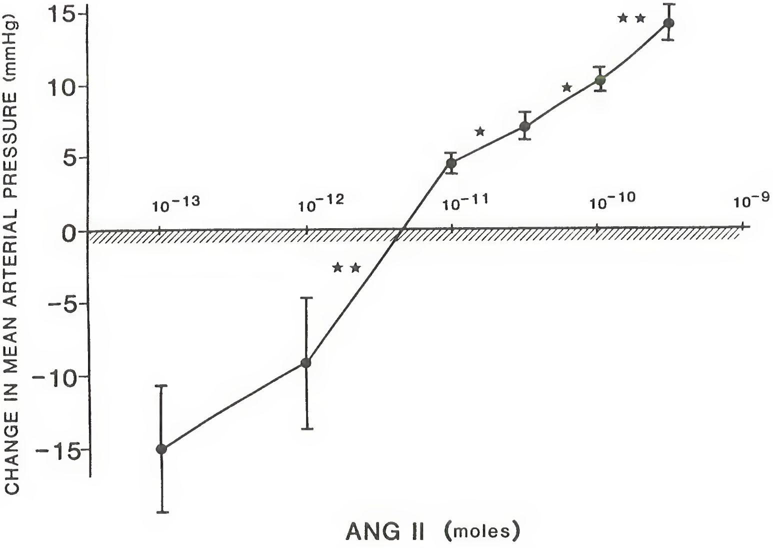
Dose Response. The blood pressure and heart rate responses to six doses of ANG II and the artificial CSF vehicle are presented in table 4-1. The lowest dose (0.1 pmole) produced a significant ($P < .01$) decrease in MAP (-14.8 ± 4.5 mmHg) but no significant change in HR. A dose of 1 pmole also produced a significant ($P < .05$) decrease in MAP accompanied by a highly variable decrease in HR. In general, the two low doses of ANG II produced a decrease in MAP but the response was highly variable as witnessed by the large standard error of the changes. The next largest dose (10 pmole) and all remaining doses produced increases in MAP. All were statistically significant at $P < 0.01$ when compared to the vehicle. The response to the three largest doses was not significantly different from that found previously in animals tested under the same conditions. Figure 12 illustrates the biphasic nature of the MAP effect

TABLE 4-1. THE HEART RATE AND BLOOD PRESSURE RESPONSE TO DIFFERENT DOSES OF ANGIOTENSIN II MICROINJECTED INTO THE NTS.

	BLOOD PRESSURE (mmHg)				HEART RATE (beats/min)			
ANG II Dose	Resting MAP	Treatment MAP	Change in MAP	P value <	Resting HR	Treatment HR	Change in HR	P value <
aCSF	89.3 ± 3.4	89.4 ± 3.1	0.1 ± 0.7		327 ± 18	329 ± 12	2 ± 3	
0.1 pmole	98.1 ± 6.4	83.3 ± 8.1	-14.8 ± 4.5	.01	338 ± 22	328 ± 22	-10 ± 6	
1 pmole	92.9 ± 6.7	83.8 ± 9.0	-9.1 ± 4.6	.05	347 ± 12	346 ± 16	-1 ± 7	
10 pmole	88.0 ± 4.8	92.7 ± 4.5	4.7 ± 1.0	.01	351 ± 20	355 ± 19	4 ± 3	
50 pmole	83.5 ± 2.4	91.1 ± 3.2	7.6 ± 1.1	.01	369 ± 16	378 ± 18	9 ± 7	
250 pmole	85.2 ± 2.4	95.3 ± 2.7	10.1 ± 1.7	.01	380 ± 6	389 ± 8	9 ± 3	.05
500 pmole	86.6 ± 2.9	101.4 ± 3.8	14.8 ± 1.2	.01	388 ± 6	399 ± 5	11 ± 1	.05

Values are means ± SE. ANG II, angiotensin II; NTS, nucleus tractus solitarius; MAP, mean arterial pressure; HR, heart rate; aCSF, artificial cerebrospinal fluid. P value indicates level of statistical significance by ANOVA compared to aCSF control.

Figure 12. Mean arterial pressure (MAP) change after microinjection of angiotensin II (ANG II) into the medial nucleus tractus solitarius. Measurements are mean ± SE. Hatched area indicates the mean ± SE of the artificial cerebrospinal fluid vehicle response. All values are significantly different from controls. Asterisks indicate significance from next lowest dose. (*P < 0.05, **P < 0.01).


CHANGE IN MEAN ARTERIAL PRESSURE (mmHg)
15
10
5
0
-5
-10
-15
10^{-13}
10^{-12}
10^{-11}
10^{-10}
10^{-9}
★★
★
★
★★
ANG II (moles)

of ANG II microinjected into the NTS. Changes in heart rate were only associated with the two largest doses (250 and 500 pmole). Both produced significant ($P < 0.05$) increases when compared to the artificial CSF vehicle. In general, the change in heart rate tended to follow the change in MAP over the entire dose range.

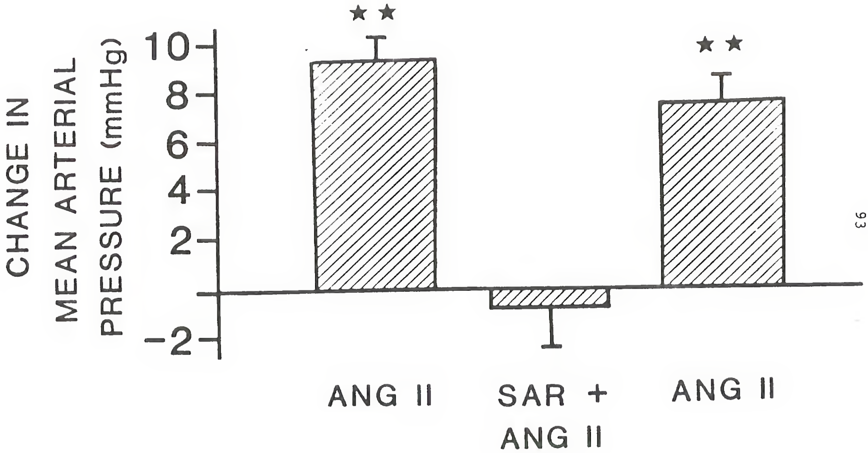
Cardiovascular actions of neuropeptides localized in NTS. The results of microinjection of a single dose of the neuropeptides; somatostatin, bradykinin, VIP, substance P, oxytocin, and vasopressin into the NTS are shown in table 4-2. Of the peptides tested only substance P (3.2 ± 0.7 mmHg), oxytocin (6.2 ± 1.4 mmHg) and vasopressin (27.0 ± 3.0 mmHg) produced changes in MAP and only substance P (13 ± 3 beats/min) and vasopressin (24 ± 8 beats/min) were effective in changing heart rate. The ability of substance P to alter MAP was apparently secondary to the increase in heart rate since the magnitude of the pressor response was small while the increase in heart rate was relatively large. Vasopressin was highly effective in increasing both MAP and HR, and oxytocin was effective at the dose tested in producing a significant pressor response even in the face of decreasing heart rate. Matsuguchi et al. (1982) have previously reported that vasopressin microinjected into the NTS produced increases in MAP and HR and that oxytocin in larger doses increases MAP but at smaller doses produced no effect. The ability of oxytocin to produce these changes

TABLE 4-2. THE BLOOD PRESSURE AND HEART RATE RESPONSE TO VARIOUS NEUROPEPTIDES IN THE NTS.

	BLOOD PRESSURE (mmHg)				HEART RATE (beats/min)			
Injectate	Resting MAP	Treatment MAP	Change in MAP	P value	Resting HR	Treatment HR	Change in HR	P value
Somatostatin	94.5 ± 6.8	93.3 ± 7.5	-1.1 ± 1.1		317 ± 58	310 ± 61	-7 ± 7	
Bradykinin	97.8 ± 6.2	95.3 ± 6.5	-2.5 ± 2.5		337 ± 27	332 ± 24	-5 ± 3	
VIP	80.2 ± 2.6	80.5 ± 1.2	0.3 ± 1.5		268 ± 22	263 ± 23	-5 ± 3	
Substance P	92.7 ± 1.2	95.9 ± 1.0	3.2 ± 0.7	.05	330 ± 28	342 ± 26	13 ± 3	.02
Oxytocin	97.2 ± 2.1	103.4 ± 2.5	6.2 ± 1.4	.05	383 ± 24	373 ± 23	-10 ± 10	
Vasopressin	96.3 ± 4.4	123.3 ± 6.7	27.0 ± 3.0	.01	303 ± 21	327 ± 19	24 ± 8	.01

Values are mean ± SE for 6 animals/group. The dose of each peptide equals 0.5 nmole. NTS, nucleus tractus solitarius; MAP, mean arterial pressure; HR, heart rate; VIP, vasoactive intestinal peptide. P value indicates level of statistical significance compared to aCSF vehicle control.

Figure 13. Mean arterial pressure change as a result of injection of 100 pmole angiotensin II (ANG II) into the medial nucleus tractus solitarius (NTS) before, during, and after NTS blockade of ANG receptors with saralasin (1 nmole). Sample size equals 6 in each case. Values are mean ± SE. Significant difference from control injection of vehicle: $^{**}P < 0.01$.


CHANGE IN
MEAN ARTERIAL
PRESSURE (mmHg)
10
8
6
4
2
-2
★★
★★
ANG II
SAR +
ANG II
ANG II

may have been nonspecific since the effect had no relation to dose in the larger dose range.

Blockade of angiotensin and vasopressin receptors in NTS. Figure 13 demonstrates the blood pressure changes associated with injection of ANG II (100 pmole) before, during, and after blockade of angiotensin receptors within the NTS. Preblockade injections of 100 pmole ANG II increased MAP from 96.8 ± 2.3 to 106.2 ± 2.3 mmHg ($P < .01$) and heart rate from 369 ± 3 to 377 ± 4 beats/min ($P < .05$). Injection of this dose of saralasin does not alter resting arterial pressure or heart rate. At larger doses however, saralasin demonstrates agonistic properties in the NTS. After saralasin blockade, a similar injection produced no change in MAP (-0.8 ± 1.6 mmHg) or heart rate (-5 ± 8 beats/min). Sixty minutes after angiotensin blockade, the same dose of ANG II produced an increase in MAP from 95.6 ± 2.0 to 103.0 ± 1.7 mmHg but no change in heart rate (367 ± 15 vs. 378 ± 15 beats/min). The magnitude of the pressor response before and after angiotensin blockade was not significantly different. Figure 14 demonstrates the inability of the vasopressin antagonist to affect the angiotensin pressor effect in the NTS. The magnitude of the response was not significantly different before versus after vasopressin blockade at each dose; 50 pmole (5.2 ± 0.6 vs. 4.8 ± 0.6 mmHg), 100 pmole (6.7 ± 1.2 vs. 6.7 ± 0.4 mmHg) and 250 pmole (10.1 ± 1.7 vs. 12.1 ± 1.2 mmHg).

Pretreatment of the NTS with the ANG II antagonist, saralasin (1 nmole) did not attenuate the vasopressin pressor effect at a single dose (10 pmole). Before saralasin blockade, the vasopressin microinjection produced an increase in MAP of 11.8 ± 1.9 mmHg. During and after blockade the response was not significantly different yielding increases in MAP of 13.0 ± 1.7 mmHg and 12.3 ± 1.1 mmHg respectively. These results clearly demonstrate that ANG II and vasopressin are acting at independent receptors to produce a pressor response at the NTS.

Pressor response of ANG II in the SHR. Intravenous administration of phenylephrine produced the expected dose-related increase in arterial pressure (Figure 15) and reflex decrease in heart rate. Analysis of variance revealed that the strain of rat was not a factor in determining the magnitude of the pressor effect. This is different than reports in conscious animals, where peripheral vasoconstrictors exhibit exaggerated pressor responses in SHR (Okamoto et al., 1966). There was a significant effect of species on the ANG II response in the NTS. At the lowest dose of ANG II tested, 50 pmole, there was no significant difference in the pressor response between the SD-WKY-SHR animals. However as shown in Figure 16, at all the remaining doses tested the SHR group had significantly larger responses than either the SD or WKY animals. This was also true of the heart rate changes.

Figure 14. Lack of attenuation of the angiotensin II pressor response by prior injection of the vasopressin antagonist $d(CH_2)_5Tyr(Me)$ arginine vasopressin (1 ug) into the nucleus tractus solitarius (NTS). Sample size equals 6 for each point. Values are mean ± SE. Circles indicate preblockade injections and triangles indicate postblockade injections.

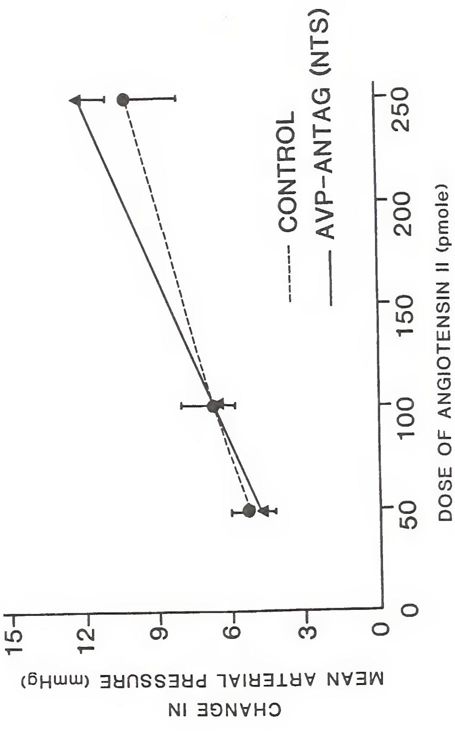
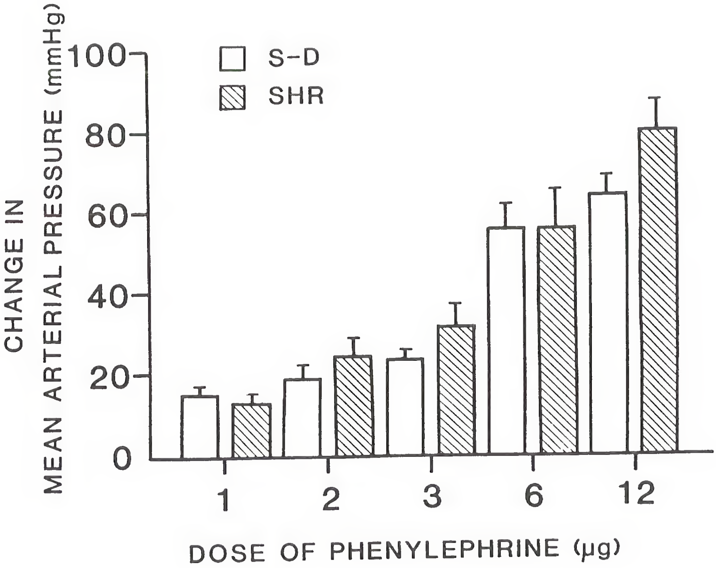

CHANGE IN
MEAN ARTERIAL PRESSURE (mmHg)
15
12
9
6
3
0
0
50
100
150
200
250
DOSE OF ANGIOTENSIN II (pmole)
CONTROL
AVP-ANTAG (NTS)

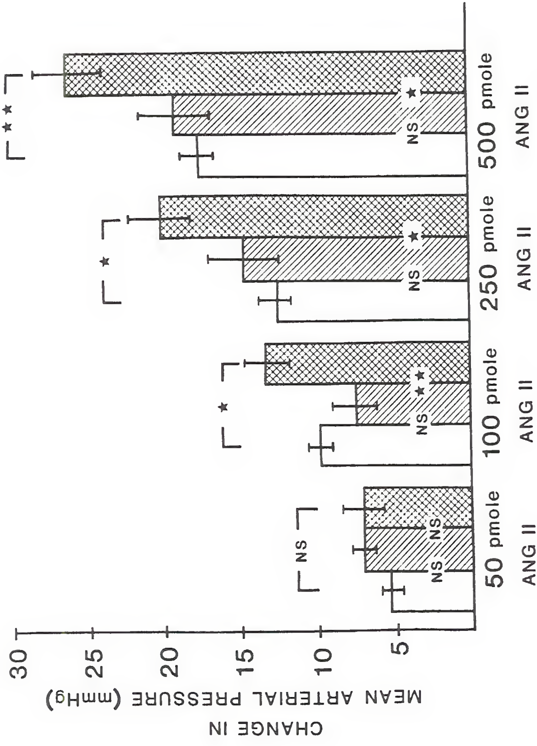
Figure 15. Change in mean arterial pressure (mmHg) produced by injecting graded doses of phenylephrine (1-12 ug) intravenously in two strains of rat: S-D, Sprague-Dawley; SHR, spontaneously hypertensive rat. There was no significant difference in the magnitude of the pressor response between the two groups.


S-D
SHR
CHANGE IN
MEAN ARTERIAL PRESSURE (mmHg)
100
80
60
40
20
0
1
2
3
6
12
DOSE OF PHENYLEPHRINE (μg)

While the SD group demonstrated an increase in heart rate only at the two highest doses and the WKY group at none of the doses, the SHR animals exhibited a significant ($P < 0.5$) increase in heart rate at all four doses when compared to the artificial CSF vehicle. However the magnitude of the tachycardia in the SHR was not significantly greater at 250 and 500 pmole than the SD animals, demonstrating an increase in the threshold sensitivity but not in the magnitude of the heart rate response. This is unlike the pressor response which exhibited an increase in magnitude of response at larger doses, although the percentage of increase did not appear to be dose-dependent.

Histology. Microscopic examination of the brain stem revealed that the dye injected into the medial NTS apparently followed the structure of the NTS by spreading predominately in a rostrocaudal direction. The medial NTS was stained in all animals. Additionally, dye stained a portion of the commisural subnucleus in approximately one-half of the animals. In only two animals, a portion of the lateral NTS or a small number of cells on the border of the NTS and the dorsal motor nucleus of the vagus (DMV) were stained.

Figure 16. Change in mean arterial pressure (mmHg) produced by injecting 4 doses of angiotensin II (ANG II) into the medial nucleus tractus solitarius of three strains of rat: Sprague-Dawley, open bars; Wistar-Kyoto, hatched bars; Spontaneously Hypertensive Rats, cross-hatched bars. Sample size equals 6 for each group at each dose. Values are mean ± SE. Asterisks indicate level of significance by analysis of variance, $*P < 0.05$, $**P < 0.01$.


CHANGE IN
MEAN ARTERIAL PRESSURE (mmHg)
30
25
20
15
10
5
50 pmole
ANG II
100 pmole
ANG II
250 pmole
ANG II
500 pmole
ANG II
NS
NS
NS
NS
*
**
NS
*
*
NS
*
NS
**
*

Discussion

There is direct evidence for a role of the brain renin-angiotensin system in the maintenance of hypertension in SHR. For example, Phillips and coworkers (1977b) were able to lower the blood pressure of SHR by central administration of saralasin. This demonstrates that the brain renin-angiotensin system alone is capable of increasing blood pressure to a hypertensive state, since the blood pressure of WKY animals was not affected. Also, the SHR appears to possess a hyperactive brain renin-angiotensin system which contributes to the maintenance of hypertension (Phillips et al., 1977b).

The present study shows that the SHR is hyperresponsive to NTS application of ANG II. Increased ANG II-immunoreactivity in the brain of SHR has been documented (Phillips et al., 1982a). One site of increase is the NTS. Increased levels of ANG II as determined by radioimmunoassay have also been reported in the brains (Ganten et al., 1983) and specifically in the brain stem of SHR (Hermann, 1983). Classical ligand-receptor interactions suggest that increased levels of ANG II would decrease the number of it's receptors yielding an attenuation of the response in SHR, not an increase as seen. It is not clear that central or peripheral ANG II follows this model for regulating it's receptor number. Increased levels of circulating ANG II have been demonstrated to produce either an increase (Aguilera et al., 1980), decrease (Gunther et al., 1980), or no change (Douglas and Brown, 1983) in receptor number in different peripheral tissues, and the effect of increased brain ANG II on it's receptors is presently unclear. The increased pressor responsiveness to both intraventricular (Hoffman et al., 1977) and NTS administration of ANG II concomitant with increased endogenous levels is therefore not improbable. Taken together, these findings add evidence for a role of brain ANG II in the pathophysiology of hypertension in the SHR.

The biphasic nature of the dose response curve for ANG II in normotensive animals poses several interesting questions. First, what is the true physiologic role of endogenous ANG II in the NTS? Secondly, by what mechanism or mechanisms are these opposite effects being produced? The close proximity of the NTS to the DMV and evidence that stimulation of either nucleus decreases blood pressure belie the fact that the mechanism for the decrease in blood pressure is entirely different in these two nuclei. The cell bodies of cardioinhibitory parasympathetic neurons lie within two brain-stem nuclei, the DMV and the nucleus ambiguus (Nosaka et al., 1979). Stimulation of the DMV produces bradycardia and a secondary fall in blood pressure. Stimulation of the NTS, also decreases blood pressure but through direct sympathetic inhibition resulting in a primary decrease in blood pressure. Whether the low doses of ANG II used in this study act by one mechanism or the other, was not addressed. Preliminary reports by Diz et al. (1984) indicate that very low doses of ANG II injected into the DMV elicit a decrease in both MAP and heart rate. It is possible that ANG II injected into the NTS occasionally diffused to cells within the DMV, explaining the highly variable responses at low doses. Histology indicated that occasionally a few cells within the DMV were stained with dye injected into the NTS. The fact that only a very few cells in the DMV are reached by the injectate, combined with the very low dose of ANG II, suggest that the DMV contains cells which are highly sensitive to ANG II. In these studies, the decreases in blood pressure were not associated with large decreases in HR, as seen by Diz et al. with injections into the DMV. At larger doses a consistent NTS pressor effect may override the occasional depressor effect caused by spread to the DMV.

It may be speculated that low doses of ANG II are causing the local release of norepinephrine within the brain stem, a compound with acute depressor effects in the NTS (Zandberg et al., 1979). Peripheral ANG II is known to facilitate release of norepinephrine during stimulation of postganglionic autonomic nerves, possibly through activation of presynaptic receptors (Zimmerman, 1978). Recent studies indicate that central injection of ANG II causes an increase in catecholamine activity in specific brain stem and hypothalamic nuclei (Sumners and Phillips, 1983). Thus, at low concentrations, ANG II may act to increase the level of tonic catecholamine activity at depressor sites within the brain stem and thereby elicit depressor effects.

Unlike the responses at lower doses, the pressor responses showed relatively little variability. In addition the two largest doses were accompanied by increases in HR not decreases. From these studies, it cannot be stated which dose range or which effect represents the physiologic role of ANG II in the NTS. The physiologic role of angiotensin may represent both pressor and depressor action which depend on both the concentration of ligand present and the physiological state of the animal. Although autoradiographic techniques have localized specific angiotensin receptors within the NTS (Mendelsohn et al., 1984), the absence of kinetic studies and studies concerning the concentrations of these ligands at their receptor sites have made determination of appropriate doses difficult.

The demonstration that saralasin specifically inhibits the pressor response to ANG II but not to vasopressin and the study showing that vasopressin antagonists have no effect on the ANG II pressor effect make two important points. First, it clearly demonstrates that the ANG II pressor effect is dependent on specific binding of angiotensin receptors within the NTS. Since ANG II and vasopressin produce similar cardiovascular effects in the NTS, it was possible the final mediator of the ANG II pressor effect was vasopressin, or the final mediator of the vasopressin response was ANG II. Although the postreceptor pathway mediating these pressor effects in the NTS is unknown, the studies using the opposite antagonist demonstrate that neither of these two peptides is dependent on the release and binding of the other. It is possible that a parallel pressor system for ANG II and vasopressin exists within the NTS and that a common efferent pathway is involved. Rapid tachyphylaxis to vasopressin occurs in the NTS. There is no evidence for attenuated responses after several applications of ANG II. Had the ANG II pressor effect been mediated through vasopressin release or vasopressin receptors, tachyphylaxis to ANG II would have been expected.

The comparison of other neuropeptides in this study emphasizes the uniqueness of ANG II and vasopressin. Their singular ability to increase blood pressure is in contrast to the wide variety of substances which decrease blood pressure at the NTS. The lack of pressor or depressor activity by the other neuropeptides does not preclude their having a role in cardiovascular regulation at the NTS. It does demonstrate that they do not directly effect the activity of primary efferents to the sympathetic or parasympathetic nervous system within the same dose range as either vasopressin or angiotensin. These neuropeptides may have a role in modulation of descending hypothalamic fibers to the NTS, or may modulate changes in the activity of the primary baroreceptor afferents, by acting as interneurons in the baroreflex arc. Their physiologic role may only be elucidated by studying their effect during changes in baroreflex activity. For example, neonatal capsaicin treatment results in the degeneration of primary sensory afferent fibers and substance P microinjection into the NTS of such animals produces an acute increase in blood pressure (Carter and Lightman, 1983). Since the NTS is not only cardiovascular in nature, these peptides may have a role in integration of respiratory or gustatory responses.

The common increase in responsiveness of SHR to intraventricular and NTS administration of ANG II may be due to a lack of baroreflex inhibition. The primary level at which the heightened responsiveness occurs is not known. The possibilities include an increase at the level of the NTS, or increases at the level of the effector sites, possibly the sympathetic ganglia. An attempt was not made to determine the mechanism of increased responsiveness in the SHR group. However, the experiment with phenylephrine eliminates the possibility that SHR are merely hyperresponsive to all pressor stimuli, at least in the urethan-anesthetized state. Although SHR have been shown to exhibit exaggerated pressor responses to peripheral vasoconstrictors in the intact state, Okamoto et al. (1966) have shown that pithing eliminates the increased response to norepinephrine suggesting a central site of hyperreactivity. The urethan anesthetic appears to have blunted the increased response to phenylephrine expected in SHR. This indicates that the site of hyperresponsiveness to NTS application of ANG II is of central origin and that in the absence of anesthetic, the increased response of SHR to ANG II in the NTS would have been exaggerated to a greater degree. While the SD animals increased HR only to the two largest doses of ANG II tested, a significant increase in the HR was observed at all doses tested in the SHR.

In summary, of the peptides localized in the NTS, ANG II and vasopressin appear to be unique in their ability to produce an increase in MAP at the NTS. They act at independent receptor sites as blockade of one system has no effect on the potency of the other. Both peptides produce not only an increase in MAP but also consistent increases in HR. This alone suggests inhibition of the baroreflex at the level of the NTS. Since, brain angiotensin has been implicated in the maintenance of hypertension in the SHR, the increased responsiveness of the SHR to ANG II in the NTS may represent one of the primary sites of action of endogenous ANG II which result in increased sympathetic outflow in the SHR (Iriuchijima, 1973).

CHAPTER 5
ATTENUATION OF BAROREFLEX FUNCTION MEDIATED BY ANGIOTENSIN AT THE NTS

Introduction

Most of the studies investigating the role of brain angiotensin in the regulation of blood pressure (Ganten et al., 1975b) and water balance (Fitzsimons, 1978) have used central injection of the octapeptide ANG II itself. The metabolism of ANG II in the brain and biological activity of possible metabolites has received much less attention.

Recent studies investigating the binding of angiotensin to high affinity sites in the rat brain, have demonstrated that ANG III is a highly successful ligand for angiotensin receptors in the central nervous system (Mendelsohn et al., 1984). In fact, in a competitive binding assay, ANG III and ANG II were almost equally potent in displacing a radioactive ligand, suggesting that brain ANG III may play a physiologic role. In a study on the relative pressor effects following intraventricular injection of ANG I, ANG II and ANG III, Tonnaer et al. (1982) found that all three peptides induced water intake and significant pressor responses. ANG II was the most potent in both respects followed by ANG I and ANG III in that order. The presence of ANG III in the CSF (Hutchinson et al., 1978), together with its central dipsogenic and pressor effects, may indicate a functional role for the peptide in the brain, although in most studies it has a lower activity than ANG II. Since both ANG I and ANG II have now been shown to have pressor action in the NTS, it was decided to determine whether ANG III possessed a similar ability to alter blood pressure when microinjected into the NTS.

In a previous study in this series, the role of the sympathetic nervous system in the pressor response subsequent to ANG II microinjection into the NTS was assessed by using the ganglionic blocker, hexamethonium chloride (20 mg/kg, iv). Following sympathetic blockade, the pressor response to ANG II was severely attenuated but not completely abolished. It was concluded that the sympathetic nervous system was largely responsible for the increase in blood pressure but due to a slight but significant response remaining after blockade, other mechanisms could not be ruled out. Two hypotheses were formed to address this possibility. First, it was possible that a slight hormonal component was present following hexamethonium administration. This, however, seemed unlikely since hypophysectomy or a vascular vasopressin antagonist failed to alter the NTS response of ANG II.

Secondly it was possible that blockade of the sympathetic nervous system by a single intravenous injection of hexamethonium was incomplete. The intravenous injection of hexamethonium severely decreased the resting arterial pressure. However, the blockade is relatively short acting and the blood pressure begins to recover rapidly. In fact, the resting blood pressure was allowed to return to 75% of prehexamethonium treatment (3 min) in the previous study before ANG II was given in order that the resting blood pressure was not greatly different than preblockade injections of ANG II. To clarify the situation, the procedure was repeated in this study using continuous intravenous infusion of the ganglionic blocker instead of a single injection, hoping that blockade of the sympathetic nervous system would be complete. Following the ANG II treatment, the hexamethonium infusion was discontinued and the animal's blood pressure was allowed to return to normal. The ANG II response was then repeated to test whether the ganglionic blockade had any permanent effects.

Endogenous brain ANG II (Berecek et al., 1983) and circulating angiotensin, through an action on the brain (Sweet and Brody, 1970; Clough et al., 1981), play an important role in maintaining the sensitivity of cardiovascular reflexes. Impairment of the normal reflex compensation to an acute elevation in arterial pressure, mediated through the baroreceptors, could be an important contribution to the genesis of hypertension. Antagonists of the renin-angiotensin system are used as antihypertensive agents in both man and experimental animals (Geyskes et al., 1976; Phillips et al., 1977b). The fact that angiotensin converting inhibitor does not cause a reflex tachycardia as it lowers blood pressure (Phillips et al., 1977b; Berecek et al., 1983) in hypertensive animals is evidence that, in addition to vasoconstriction, ANG II must have other physiological actions in the reflex control of blood pressure. If the baroreceptors were properly exerting an inhibitory effect on the vasomotor centers, hypertension should theoretically not develop.

Peripheral as well as brain ANG II are known to have a central stimulating effect on the sympathetic nervous system. Sweet and Brody (1970) have shown that ANG II has the ability to impair reflex vasodilator responses to increased blood pressure through a central effect. ANG II when given intravenously does not attenuate the reflex vasodilator response. However, intravertebral infusion of the same dose causes a significant reduction in the reflex dilation. This suggests that angiotensin might be exerting an inhibitory influence on the baroreceptor reflex through a central rather than peripheral effect.

Stein et al. (1984) have recently demonstrated that peripheral ANG II acts upon the brain to activate central systems which interfere with the baroreflex by either direct sympathetic stimulation or parasympathetic inhibition of the heart. These studies were prompted by several earlier reports demonstrating that peripheral ANG II had potentiating effects on peripheral pressor agents both in vivo and in situ (Panisset and Bourdois, 1968; Nicholas, 1970; Ichikawa et al., 1978).

Angiotensin II given intravenously has been reported to increase heart rate as well as blood pressure, or at least prevent the expected bradycardia which should accompany relatively large increases in arterial pressure. In addition, the studies presented here have shown similar heart rate changes with ANG II given into the NTS. Since the NTS is the site of termination for baroreceptor afferent fibers (Reis and Cuenod, 1965; Seller and Ilert, 1969), this suggests that the NTS may be the central site at which angiotensin interferes with the baroreflex. To test this hypothesis, reflex decreases in heart rate in response to intravenous phenylephrine were measured before and during subpressor infusions of ANG II into the NTS. This allows assessment of both baroreceptor set point and sensitivity by comparing the changes in systolic pressure versus changes in heart rate.

The unanesthetized SHR exhibits exaggerated pressor responses to peripheral vasoconstrictors (Okamoto et al., 1966) as well as intraventricular administration of ANG II (Hoffman et al., 1977), which suggests that baroreceptor function is impaired in this animal. An earlier study in this series demonstrated that NTS application of ANG II in SHR elicited a larger pressor response than in WKY controls. The peripheral renin-angiotensin system is not thought to have a role in the pathophysiology of hypertension in SHR. Plasma renin activity is no higher (Shiono and Sokabe, 1976) than WKY animals and peripheral administration of an ANG II antagonist (Mann et al., 1978) has no effect in SHR. However, increased angiotensin-like immunoreactivity (Phillips et al., 1982a) has been reported in the brain and increased levels of an angiotensin-like material have been found in the CSF of SHR compared to WKY. Additionally, intraventricular administration of saralasin (Phillips et al., 1977b; McDonald et al., 1980) or the converting enzyme inhibitor, captopril (Unger et al., 1981), substantially lowers blood pressure in the SHR suggesting that the brain angiotensin system plays a role in the maintenance of hypertension in this model. Altered baroreflex sensitivity in the SHR has been demonstrated and captopril lowers the blood pressure without increasing heart rate (Unger et al., 1981), suggesting that it may potentiate arterial baroreflexes. Berecek et al. (1983) have demonstrated that intraventricular captopril increases the sensitivity of the baroreflex in the SHR.

Their data suggest that intraventricular captopril produces its antihypertensive effects and potentiation of baroreflex sensitivity by decreasing endogenous brain ANG II. Neuroanatomical and biochemical studies show that angiotensin-like immunoreactivity and renin content are increased in SHR in the region of the NTS (Ganten et al., 1979; Phillips et al., 1982a), the first synapse of the baroreflex arc.

These data taken together with increased NTS-reactivity to ANG II in the SHR led to this investigation into the effects of NTS application of captopril on the baroreflex responses to graded doses of phenylephrine in the SHR. The goal of this study was to determine whether depletion of NTS angiotensin with captopril would alter resting arterial pressure in SHR and, as with intraventricular captopril increase the sensitivity of baroreceptor reflexes.

Methods

Male Sprague-Dawley (SD) rats (250-300 g) were used in all experiments, except where noted. SHR and WKY rats were obtained from Charles River Breeding Laboratory (Wilmington, MA.). Rats were housed in individual cages in a temperature-controlled room on a 12-h light-12-h dark cycle. All experiments were performed under urethan anesthesia (1.2-1.4 g/kg, ip). The femoral artery was catheterized with PE-50 tubing and connected via a Statham P23Gb transducer to a Gould chart recorder for continuous measurement of blood pressure and heart rate. The trachea was cannulated with curved tubing so as not to obstruct the airway when the head was flexed.

To expose the medulla, the animals were placed in a stereotaxic frame with the head flexed downward to an angle of 45 degrees. After a midline incision through the skin, the dorsal neck muscles were retracted with sutures exposing the foramen magnum. The caudal portion of the fourth ventricle was exposed by incising the atlantooccipital membrane and, when necessary, gently retracting the cerebellum with a spatula. Microinjections of all compounds were performed via double-barrel glass micropipettes (60-80 um outer tip diameter) connected by PE-10 tubing to remote Hamilton microliter syringes (no. 701N) driven by two Sage infusion pumps (model 341). When possible one barrel of the micropipette was filled with dye to mark the site of injection. The vehicle for all injections was artificial CSF. The pH of all solutions injected into the NTS was between 7.0 and 8.0 except where noted. Bolus injections into the NTS were given in a volume of less than or equal to 100 nl. At least 30 min were allowed before injections into the NTS were repeated. Each rat received injections only into the medial NTS; the coordinates 0.5 mm rostral to the caudal tip of the area postrema, 0.5 mm lateral to the midline, and 0.5 mm below the dorsal surface of the neural tissue were used.

For intravenous infusion of phenylephrine or hexamethonium, the femoral vein was catheterized. Venous catheters were constructed from PE-50 tubing and connected to a Sage infusion pump for delivery of compounds. Intravenous drugs were dissolved in 0.9% saline and freshly prepared each day.

Cardiovascular effects of ANG III in the NTS. Six SD rats were prepared as described above. One barrel of a glass micropipette was filled with the artificial CSF vehicle and the other filled with an artificial CSF solution containing 1 ug ANG III/100 nl. The micropipette was lowered into the right NTS and after stabilization of blood pressure and heart rate (approximately 15 min) the peptide studies were begun. Each animal received four doses of the peptide (100, 250, 500 and 1000 ng ANG III) in addition to the CSF vehicle. The order of injection was randomized in each case.

Effect of hexamethonium infusion on ANG II pressor response. The blood pressure and heart rate responses to 500 ng of ANG II microinjected into the NTS were tested in six SD rats that had been given the ganglionic blocker, hexamethonium chloride. A glass micropipette containing ANG II (500 ng/100 nl) dissolved in artificial CSF was lowered into the right NTS, and a control injection of 500 ng ANG II given. Thirty min after the test dose of ANG II, an intravenous infusion of hexamethonium (7.5 mg/kg/min.) was started which immediately lowered the blood pressure and heart rate. In animals in which the blood pressure began to return toward preinfusion level, the infusion rate was increased to 10 ng/kg/min. After four min of continuous infusion the blood pressure and heart rate had stabilized in all animals and the ANG II injection was repeated. Ten min after ANG II injection, the hexamethonium infusion was discontinued and the blood pressure and heart were allowed to return to normal. When the mean arterial pressure had returned to the preinfusion level, but in no case sooner than 30 min after hexamethonium discontinuation, 500 ng ANG II was again microinjected into the NTS. The cardiovascular responses during and after hexamethonium treatment were compared with preblockade values to determine whether complete ganglionic blockade affected the angiotensin response in the NTS.

Baroreflex during NTS infusion of ANG II. Six SD rats were prepared as described above including intravenous catheters. One barrel of the micropipette was filled with the artificial CSF vehicle and the other with a solution containing ANG II. The micropipette was lowered into the right NTS and blood pressure and heart rate were monitored until stable. The intravenous catheter was filled with a saline solution containing the vasoconstrictor phenylephrine (24 ug/ml). An infusion of artificial CSF (120 nl/h) into the NTS was begun and cardiovascular parameters were monitored for 10 min. Arterial pressure and heart rate responses to graded doses of phenylephrine (1-12 ug, iv) given in a random order were monitored. At least 15 min were allowed between each dose of phenylephrine. Each infusion of phenylephrine was given over 60 s, with the dose being adjusted by changing the rate of infusion. Upon completion of the last dose of phenylephrine the NTS infusion of CSF was discontinued. The rat was allowed to recover for 90 min. At the end of the recovery period, a subpressor infusion of ANG II (10 ng/kg/min) into the NTS was initiated. This dose had been determined to be slightly below the threshold necessary to increase blood pressure or heart rate during an infusion for 120 min, and was given in the same volume (120 nl/h) as the CSF control. The phenylephrine infusions were repeated as above. After the final phenylephrine dose the blood pressure and heart rate were monitored for 30 min to determine whether the angiotensin infusion had altered resting arterial pressure or heart rate. The ANG II infusion was then discontinued and the blood pressure and heart rate monitored for an additional 30 min.

Baroreflex in SHR after NTS application of captopril. Six SHR rats were prepared as above including intravenous catheters. The intravenous catheter was filled with a saline solution containing the vasoconstrictor phenylephrine (24 ug/ml). One barrel of the micropipette was filled with a solution containing the angiotensin converting enzyme inhibitor, captopril (20 ug/100 nl). Since solutions containing captopril are highly acidic (pH 3.5), the pH of the CSF control was titrated to the same hydrogen ion strength. The CSF was then loaded into the other barrel of the micropipette. The micropipette was lowered into the left NTS and blood pressure and heart rate monitored until stable. CSF (pH 3.5) in a volume of 100 nl was injected, the micropipette was removed and placed in the right NTS. Three min later, 100 nl of CSF (pH 3.5) was injected into the right NTS. The blood pressure and heart rate were monitored for 20 min to determine whether NTS application of CSF (pH 3.5) would alter resting arterial pressure in SHR. Graded doses of phenylephrine (1-12 ug, iv) were administered in a random order with at least 15 min allowed between each dose. Upon completion of the last dose of phenylephrine, the rat was allowed to recover for 60 min. At the end of the recovery period, captopril (20 ug) was injected into the right NTS. The micropipette was moved to the left NTS and three min later the same dose of captopril was injected into the left NTS. This dose of captopril when given intraventricularly has been shown to decrease blood pressure in SHR and prevent the pressor response to subsequent central injections of ANG I, but not affect blood pressure in normotensive controls (Unger et al., 1981). Blood pressure and heart rate were monitored for 20 min and the five doses of phenylephrine infusions were repeated as above.

Verification of injection sites and statistics. The injection sites were marked by injecting 100 nl of Evans blue dye. Thirty min after dye injection, the rats were perfused transcardially with 50 ml normal saline followed by 50 ml of 10% buffered formaldehyde, the brain was removed, and 40 micron coronal sections of the brain stem were cut and Luxol fast blue-cresyl violet stained for histological verification of injection site. Pulsatile arterial pressure and heart rate were recorded throughout the procedures. MAP was calculated from the pulsatile record. Change in mean arterial pressure and heart rate were measured 1 min after completion of injections into the NTS. Baroreflex sensitivity was evaluated with graded doses of phenylephrine (maximum infusion volume = 500 ul). Peak increases in systolic arterial pressure (SYS) to phenylephrine and the associated decreases in heart rate were recorded for each dose. Heart rate was converted to pulse interval by the formula: PI (in msec) = 60,000/heart rate. Therefore, the heart rate is inversely related to pulse interval. The slope for baroreceptor control of heart rate (change in PI/change in SYS) was determined for each rat by fitting a least squares regression line through the points relating change in PI to change in SYS. Individual slopes and correlation coefficients were averaged for each group of rats. Statistical comparison between the groups was performed using Student's t test. Multiple comparison of means was accomplished by the use of analysis of variance (ANOVA) followed by the Newman-Keuls test when a significant F ratio was obtained. $P < 0.05$ was considered significant. All reported values are mean ± SE.

Results

Cardiovascular effects of ANG III in the NTS. Microinjection of the CSF vehicle alone had no effect on MAP or heart rate in any of the animals tested. However, all doses of ANG III produced significant ($P < 0.05$) pressor effects and increases in heart rate. The dose of 100 ng increased blood pressure and heart rate from 104.6 ± 3.5 mmHg and 379 ± 17 beats/min to 108.8 ± 3.7 mmHg and 394 ± 16 beats/min. The 250 ng dose increased mean blood pressure from 105.8 ± 3.5 to 113.7 ± 4.0 mmHg and heart rate from 375 ± 14 to 395 ± 12 beats/min. The dose of 500 ng ANG III increased mean blood pressure from 106.2 ± 2.6 to 115.2 ± 2.6 mmHg and heart rate from 385 ± 15 to 399 ± 14 beats/min. The largest dose (1000 ng) increased mean blood pressure from 105.9 ± 3.1 to 118.9 ± 4.3 mmHg and heart rate from 373 ± 13 to 388 ± 17 beats/min. Although the heart rate increases were significant at every dose tested, there was no effect of dose on the magnitude of increase when compared by analysis of variance. A comparison of magnitude of the pressor response indicated that the 250 ng dose was significantly ($P < 0.05$) more potent than the 100 ng dose and that 1000 ng was significantly ($P < 0.05$) more potent than 500 ng. However, there was no significant difference in the magnitude of the response to 250 and 500 ng of ANG III. Figure 17 illustrates the relative potency of the three peptides ANG I, II and III in producing pressor effects in the NTS.

Effect of hexamethonium infusion on ANG II pressor response. The preblockade injection of 500 ng ANG II increased MAP by 16.6 ± 1.0 mmHg and heart rate 12 ± 2 beats/min. The resting MAP and heart rate values for the six rats were 98.7 ± 3.8 mmHg and 352 ± 24 beats/min immediately prior to the beginning of the hexamethonium infusion. The hexamethonium infusion significantly ($P < 0.001$) lowered both MAP and heart rate to 57.7 ± 6.5 mmHg and 288 ± 8 beats/min, respectively. This represents a decrease in MAP of 42.0 ± 9.5 mmHg and a decrease in heart rate of 65 ± 18 beats/min. Microinjection of 500 ng ANG II during the hexamethonium infusion did not alter either MAP (-0.17 ± 1.0 mmHg) or heart rate (-3 ± 2 beats/min). A 30 min postblockade injection of ANG II (500 ng) produced an increase ($P < 0.05$) in heart rate of 10 ± 3 beats/min and MAP of 15.7 ± 1.1 mmHg. The magnitude of the response to the pre- and postblockade injections was not significantly

Figure 17. Comparison of the mean arterial pressure change (mmHg) caused by microinjection of the three peptides, angiotensin I (ANG I), angiotensin II (ANG II) and angiotensin III (ANG III), into the nucleus tractus solitarius. Sample size is 6 for each peptide at each dose. Values are means ± SE. Significant difference from equal dose injection of adjacent peptide: *$P < 0.05$.

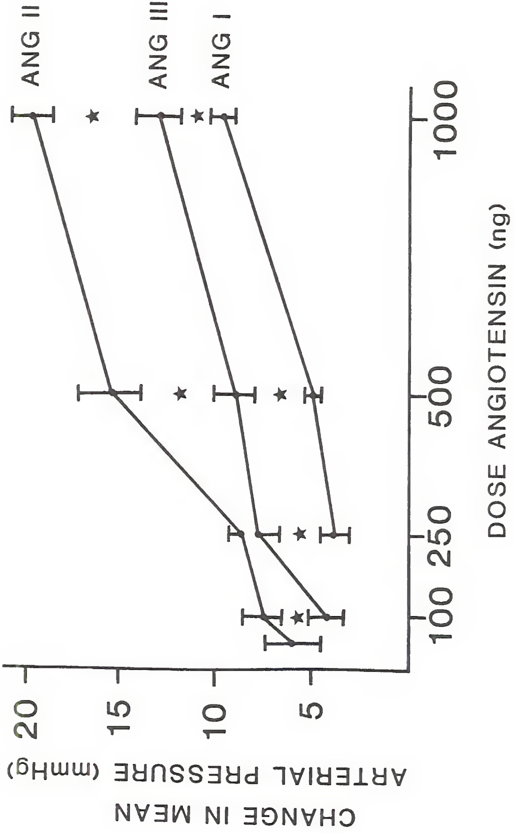

ANG II
ANG III
ANG I
CHANGE IN MEAN ARTERIAL PRESSURE (mmHg)
20
15
10
5
100
250
500
1000
DOSE ANGIOTENSIN (ng)
★

Figure 18. Mean arterial pressure change as a result of microinjection of 500 ng angiotensin II into the nucleus tractus solitarius, effect of ganglionic blockade with hexamethonium infusions (7.5-10 mg/kg/min, iv). Control, blockade, and 30 min postblockade results are shown. Sample size equals 6. Values are means ± SE.

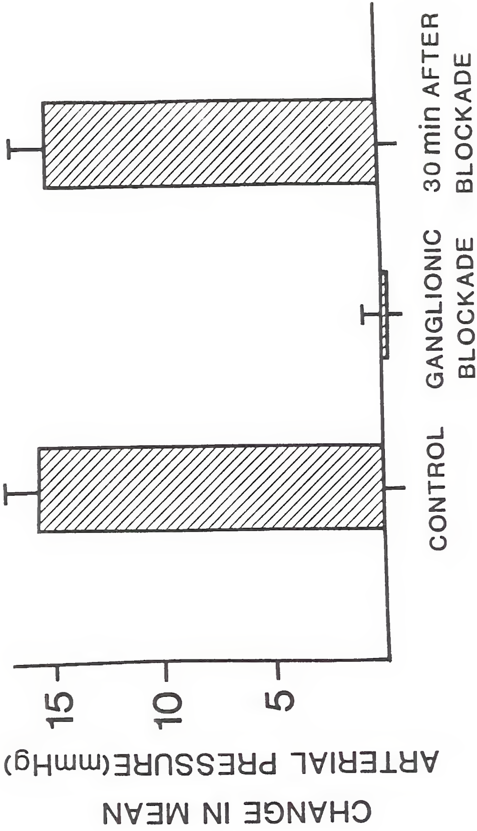
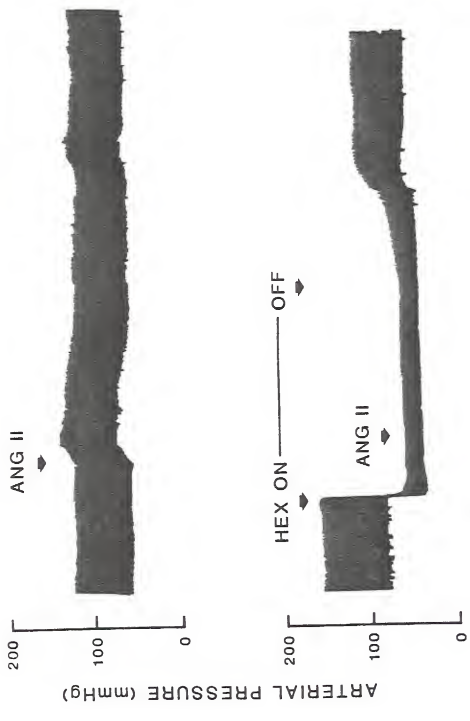

CHANGE IN MEAN
ARTERIAL PRESSURE(mmHg)
15
10
5
CONTROL
GANGLIONIC BLOCKADE
30 min AFTER BLOCKADE

Figure 19. Pulsatile pressure recording comparing the response to 500 ng angiotensin II (ANG II) before and during sympathetic blockade with hexamethonium (7.5-10 mg/kg/min, iv). Arrows, time of injection or duration of infusion.


ARTERIAL PRESSURE (mmHg)
200
100
0
ANG II
1 min
200
100
0
HEX ON
OFF
ANG II

different as shown in Figure 18. Figure 19 is a representative pulsatile recording demonstrating the preblockade ANG II response and the effect of hexamethonium on both resting pressure and the ANG II pressor response.

Baroreflex during NTS infusion of ANG II. Infusion of the CSF vehicle or ANG II did not alter either the resting arterial pressure or heart rate during the period of the phenylephrine tests. Intravenous infusion of phenylephrine produced the expected dose-dependent increase in arterial pressure and a reflex decrease in heart rate. In comparison to the CSF controls, animals treated with subpressor infusions of ANG II showed significantly greater increases in systolic pressure at all but the lowest dose of phenylephrine (Figure 20) and a significantly smaller increase in pulse interval at the two highest doses (Figure 21). The heart rate response to stimulation of the baroreceptor reflex is shown in Figure 22. Rats treated with ANG II showed significantly smaller reflex changes in heart rate in response to increases in arterial pressure induced by phenylephrine than during infusion of CSF. The points in Figure 22 represent the average change in arterial pressure and pulse interval to phenylephrine infusions. The lines represent the least squares regression through the points. It can be seen that during ANG II infusion a nonparallel shift to the right occurred in the function relating pulse interval to systolic pressure and a

Figure 20. Change in systolic pressure (mmHg) as a result of intravenous injection of graded doses of phenylephrine (1-12 ug) during nucleus tractus solitarius infusion of either artificial cerebrospinal fluid or angiotensin II (10 ng/kg/min). Sample size equals 6 at each point. Values are means ± SE. Significant difference from control at same dose: *$P < 0.05$.

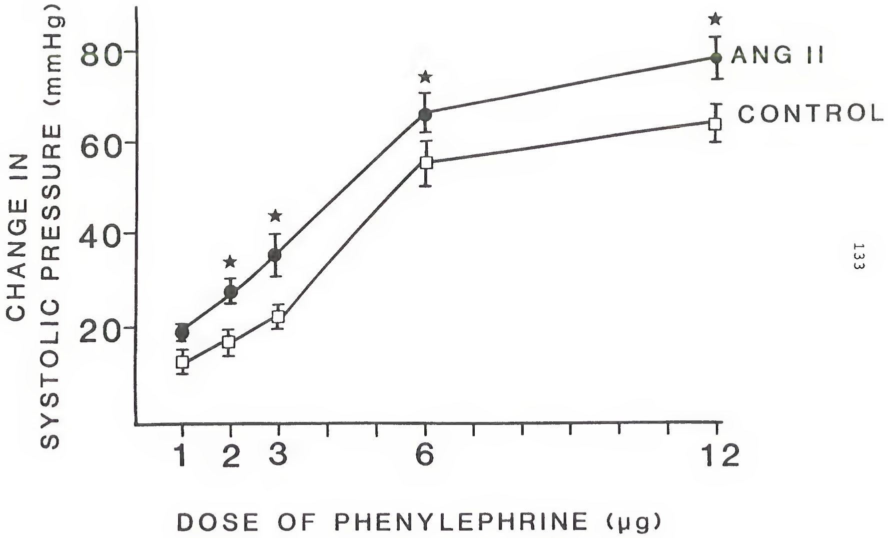

CHANGE IN SYSTOLIC PRESSURE (mmHg)
80
60
40
20
1
2
3
6
12
DOSE OF PHENYLEPHRINE (μg)
ANG II
CONTROL
★

Figure 21. Change in pulse interval (msec) as a result of intravenous injection of graded doses of phenylephrine (1-12 ug) during nucleus tractus solitarius infusion of either artificial cerebrospinal fluid or angiotensin II (10 ng/kg/min). Sample size equals 6 at each point. Values are means ± SE. Significant difference from control at same dose: $^{*}P < 0.05$.

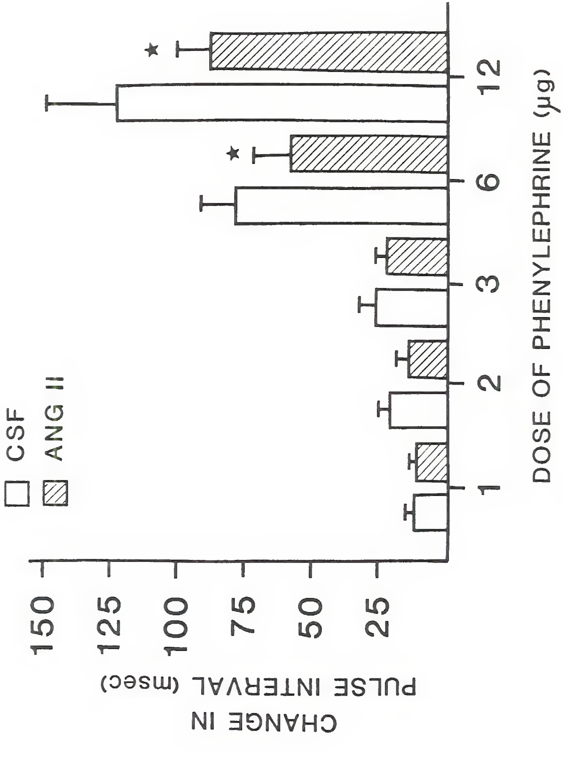
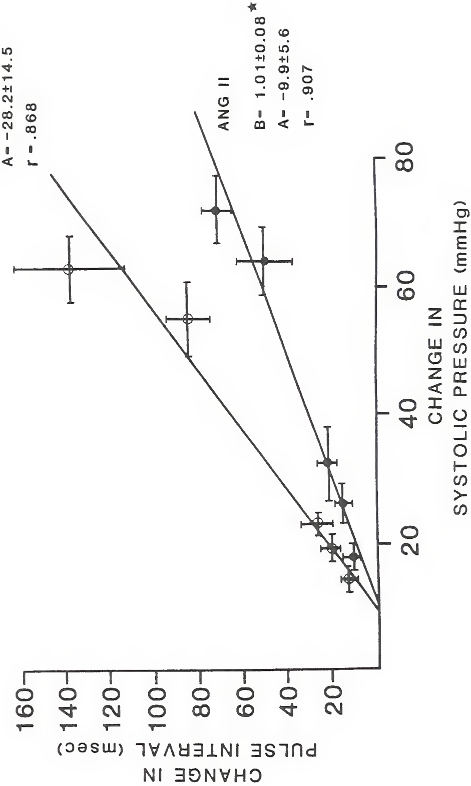

CHANGE IN
PULSE INTERVAL (msec)
150
125
100
75
50
25
CSF
ANG II
★
★
1
2
3
6
12
DOSE OF PHENYLEPHRINE (µg)

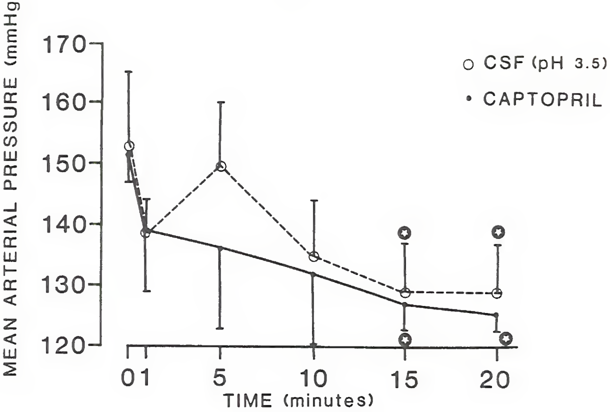
Figure 22. Function relating the change in systolic pressure (mmHg) to change in pulse interval (msec) resulting from intravenous injection of graded doses of phenylephrine (1-12 ug) during nucleus tractus solitarius infusions of either artificial cerebrospinal fluid or angiotensin II (10 ng/kg/min). Sample size equals 6 at each point. Values are means ± SE. B, slope of least squares regression line; A, y-axis intercept; r, coefficient of correlation. Significant difference from control: $^{*}P < 0.05$.


CONTROL
B = 2.41±0.51
A = -28.2±14.5
r = .868
ANG II
B = 1.01±0.08 ★
A = -9.9±5.6
r = .907
CHANGE IN
PULSE INTERVAL (msec)
160
140
120
100
80
60
40
20
20
40
60
80
CHANGE IN
SYSTOLIC PRESSURE (mmHg)

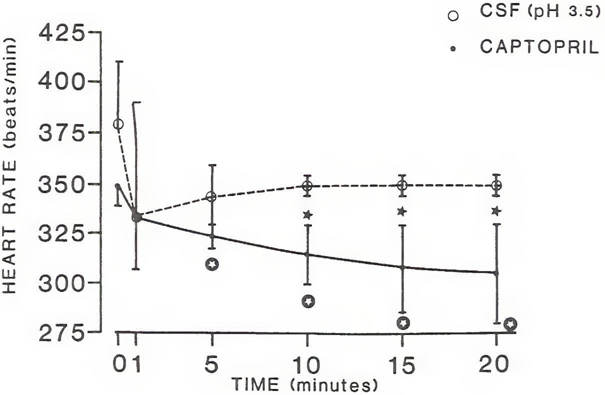
significant ($P < 0.05$) decrease in the slope (sensitivity) for baroreflex control of pulse interval. This would be expected due to the concomitant increase in systolic pressure change (Figure 20) and decrease in pulse interval change (Figure 21) on a per ug phenylephrine basis in the ANG II-treated animals. The sensitivity of the reflex during CSF treatment (2.41 ± 0.51, $r = 0.868$) was more than 2 times greater than that during ANG II treatment (1.01 ± 0.08, $r = 0.907$).

Baroreflex in SHR after NTS application of captopril. Microinjection of captopril and the CSF (pH 3.5) vehicle caused a significant ($P < 0.05$) decrease in resting mean pressure (Figure 23) at the 15- and 20-min time point. At no time was the MAP significantly different between the two treatments. Heart rate which tended to be lowered by both treatments was only significantly altered by the captopril treatment after the 1 min time period. The captopril treatment also produced significantly ($P < 0.05$) lower heart rate than the CSF (pH 3.5) treatment at the last three time periods (Figure 23). In comparison to the CSF (pH 3.5) treatment, the animals, when treated with captopril exhibited significantly larger increases in systolic pressure at the two smallest doses of phenylephrine (Figure 24). The reflex change in pulse interval was not significantly different during the two treatments at any dose of phenylephrine, except the largest (Figure 24). The

Figure 23. Mean arterial pressure (mmHg) and heart rate (beats/min) as a function of time following bilateral nucleus tractus solitarius injections of either artificial cerebrospinal fluid (CSF) titrated to pH 3.5 or captopril (20 ug/side). Sample size equals 4 at each point. Values are means ± SE. Open stars indicate significant difference ($P < 0.05$) from preinjection values. Closed stars indicate significant difference ($P < 0.05$) between CSF and captopril treatment.

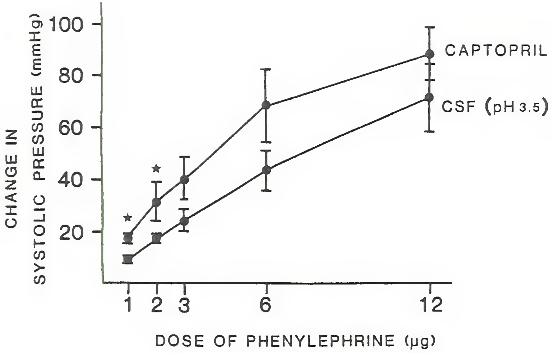


MEAN ARTERIAL PRESSURE (mmHg)
170
160
150
140
130
120
○ CSF (pH 3.5)
• CAPTOPRIL
0
1
5
10
15
20
TIME (minutes)



HEART RATE (beats/min)
425
400
375
350
325
300
275
○ CSF (pH 3.5)
• CAPTOPRIL
0
1
5
10
15
20
TIME (minutes)

Figure 24. Change in systolic pressure (mmHg) and pulse interval (msec) as a result of intravenous injection of graded doses of phenylephrine (1-12 ug) following bilateral nucleus tractus solitarius injection of either artificial cerebrospinal fluid (CSF) titrated to pH 3.5 or captopril (20 ug/side). Sample size equals 4 at each point. Values are means ± SE. Significant difference from control: $^*P < 0.05$.


CHANGE IN SYSTOLIC PRESSURE (mmHg)
100
80
60
40
20
CAPTOPRIL
CSF (pH 3.5)
*
*
1
2
3
6
12
DOSE OF PHENYLEPHRINE (µg)

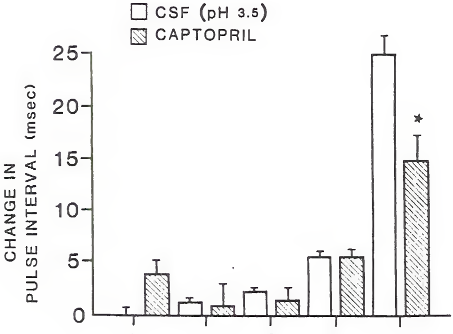
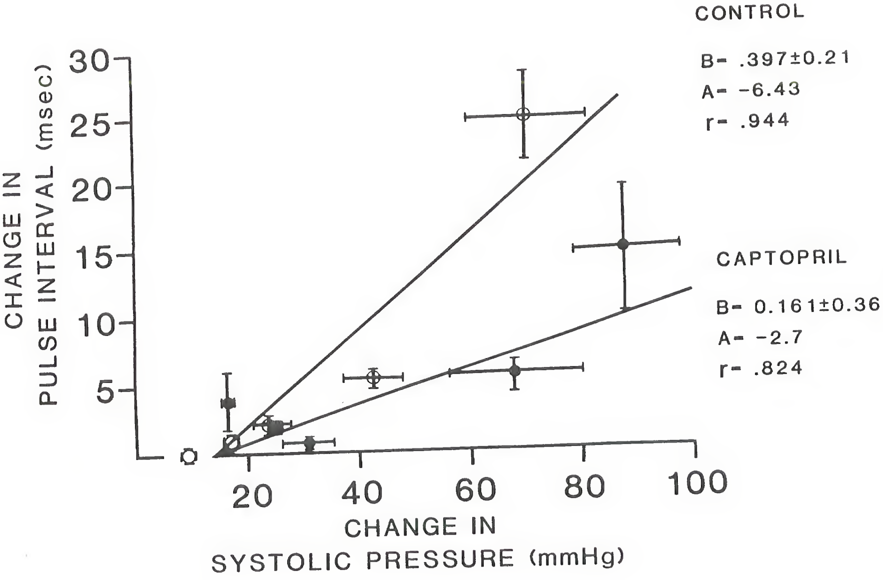

CSF (pH 3.5)
CAPTOPRIL
CHANGE IN PULSE INTERVAL (msec)
25
20
15
10
5
0
*
1
2
3
6
12
DOSE OF PHENYLEPHRINE (µg)

Figure 25. Function relating the change in systolic pressure (mmHg) to change in pulse interval (msec) resulting from intravenous injection of graded doses of phenylephrine (1-12 ug) after bilateral nucleus tractus solitarius injection of either artificial cerebrospinal fluid titrated to pH 3.5 (control) or captopril (20 ug/side). Sample size equals 4 at each point. Values are means ± SE. B, slope of least squares regression line; $\overline{A}$, y-axis intercept; r, coefficient of correlation.


CONTROL
B= .397±0.21
A= −6.43
r= .944
CAPTOPRIL
B= 0.161±0.36
A= −2.7
r= .824
CHANGE IN
PULSE INTERVAL (msec)
30
25
20
15
10
5
0
20
40
60
80
100
CHANGE IN
SYSTOLIC PRESSURE (mmHg)

points in Figure 25 represent the average change in systolic pressure and pulse interval to five doses of phenylephrine. The lines represent the least squares regression through the points. There did not appear to be any significant shift in either the slope (sensitivity) or the intercept (set point) in the function relating pulse interval to systolic pressure.

Discussion

In this series of experiments, ANG III is added to the unique list of pressor agents in the NTS. ANG II, however, retains its role as the most potent of the angiotensin family; followed by ANG III and then ANG I. The results indicate that direct injection of ANG III into the NTS produces a consistent, significant, dose-related pressor response over the dose range 100-1000 ng as well as significant increases in heart rate at each dose.

In this study, the mechanism by which ANG II causes an increase in blood pressure in the NTS is clarified. The pressor response arises solely from an increase in sympathetic flow. Ganglionic blockade with hexamethonium infusion completely abolished any pressor or heart rate response to ANG II. The slight pressor response after a single hexamethonium injection reported earlier was no longer present. This eliminates the possibility that a slight hormonal component may be present in the response. These results are in complete agreement with the earlier reports indicating that hypophysectomy or vasopressin blockade did not attenuate the ANG II response. Therefore, sympathetic activation must be considered solely responsible for the pressor effect of ANG II in the NTS. This is different from the pressor response to intraventricular injections of ANG II that have been demonstrated to involve a major contribution from pituitary release of vasopressin as well as sympathetic activation (Severs et al., 1967; Severs et al., 1970; Buckley, 1972; Severs and Daniels-Severs, 1973; Keil et al., 1975).

The ability of ANG II to alter baroreflex function at the NTS is clearly demonstrated in these studies. Earlier studies where single injections of ANG II into the NTS increased both arterial pressure and heart rate suggested that some deficit was present in the baroreflex arc. However, these studies directly demonstrate that decreased baroreflex sensitivity occurs when normotensive rats are treated with ANG II into the NTS and that the effect is attributable to ANG II rather than an alteration in baseline arterial pressure per se. The results are in agreement with a recent study by Matsuo et al. (1981), where subpressor infusions of ANG II into the cerebral ventricles increased the pressor response to intravenous infusions of ANG II.

These new data suggest that endogenous angiotensin may have an antagonistic role in the baroreceptor-mediated inhibition of autonomic nerve function. The shift in baroreflex sensitivity can be seen in the exaggerated pressor response to graded doses of phenylephrine. The concomitant inhibition of reflex slowing of the heart rate to phenylephrine produced a decrease in the slope of function relating change in systolic pressure to change in pulse interval (Figure 22). A decrease in slope of this function represents a decrease in baroreflex sensitivity. Since both experimental and genetic forms of hypertension have been associated with reduced baroreflex sensitivity, these new data suggest that endogenous brain angiotensin may act at the NTS to prevent reflex compensation to the elevated arterial pressure in these animals. Indeed, Berecek et al. (1983) were able to reverse decreased baroreflex sensitivity in SHR by chronic intraventricular administration of captopril. It was these data which prompted the present study using captopril in the NTS of SHR.

Injection of captopril into the NTS was complicated by the fact that solutions containing captopril are highly acidic. This necessitated titration of the CSF vehicle to the same pH (3.5). As a result microinjection of both the vehicle and captopril had noticeable cardiovascular effects. Captopril reduced both mean arterial pressure and heart rate. The CSF vehicle significantly reduced mean arterial pressure but heart rate also tended to be lower. This caused a significant difference in the resting heart rate when the phenylephrine studies were initiated. The lower resting heart rate caused by captopril treatment might be an indication of increased parasympathetic tone to the heart prior to the phenylephrine studies. A lack of reflex inhibition could cause the slightly larger pressor responses in the captopril group (Figure 24) and would not be totally unexpected if the parasympathetic tone to the heart was already elevated relative to controls. Reflex slowing of the heart was significantly attenuated at the largest dose of phenylephrine by the captopril treatment. This might also be due to the lower resting heart rate during captopril treatment. As a result of the marginal differences in response to phenylephrine, the slope of the function relating change in systolic pressure to change in pulse interval was not significantly changed indicating that the captopril treatment did not affect the sensitivity of the baroreflexes. Thus, the sensitivity (slope) of the baroreflex in SHR was not increased by NTS application as occurred with intraventricular administration (Berecek et al., 1983).

The studies using captopril in the NTS of SHR test the hypothesis that ANG II functions at the NTS to regulate the baroreflex and that increased levels are mediating the disfunction of baroreflexes in SHR. There are several possible explanations as to why the expected increase in baroreflex sensitivity following captopril treatment was not found. One possibility is that the effect of injecting highly acidic solutions into the NTS is masking any effect of the captopril. This seems to be likely due to the prominent cardiovascular effects of the pH 3.5 vehicle. Another possibility is that a larger dose of captopril is necessary to deplete endogenous levels of ANG II in the NTS. Although the dose used in this study has been shown to effectively block conversion of exogenously applied ANG I to ANG II within the cerebral ventricles, it is not known at what dose captopril completely depletes brain ANG II levels. A third and likely possibility is that brain ANG II acting at the NTS is not solely responsible for baroreceptor disfunction in the SHR.

One interesting observation is the relative slope of the baroreflex curves for normotensive and SHR animals (Figures 22 and 25). SHR have been demonstrated to possess an impaired baroreflex and this fact is confirmed in these studies. Compared to normotensive untreated animals, the SHR exhibit a decreased slope (sensitivity) in the baroreflex function, as expected.

In summary, microinjection of ANG III into the NTS produces an immediate increase in arterial pressure. The potency of this response is less than that of ANG II. The pressor response of angiotensin in the NTS appears to be solely mediated through the sympathetic nervous system. An ability of ANG II to alter baroreceptor reflexes directly at the NTS has been reported for the first time. This property of ANG II is not surprising in light of it's ability to directly increase sympathetic tone. In order for any substance to produce an increase in blood pressure through a central mechanism, it must be able to either increase sympathetic tone or cause the release of a peripheral pressor agent to such a degree that baroreflex inhibition of heart rate is not fully capable of compensation. This is true of ANG II and in the case of ANG II, this substance can act at the NTS to not only prevent reflex bradycardia, but to actually increase heart rate concomitant with increased blood pressure. In light of this evidence, the view that brain ANG II and ANG II-related peptides may be important in central mechanisms of blood pressure regulation is strengthened. In addition, brain ANG II may be implicated in the pathophysiology of hypertension by an action at the NTS impairing the function of the baroreflex arc.

CHAPTER 6
GENERAL SUMMARY

In recent years, it has become increasingly clear that angiotensin II functions not only as a circulating peptide in the classical sense, but is also present in specific areas of the brain, where it may function independently from the circulating hormone.

Not only is there general agreement that a renin-angiotensin system indeed exists in the brain, but specific receptors for ANG II have been found to be located in discrete cardiovascular control areas such as the nucleus tractus solitarius. Furthermore, ANG II is known to produce an increase in blood pressure partially through stimulation of the sympathetic nervous system. The mechanism of this central sympathetic activation, however, has not been elucidated and the site of the central ANG II receptors responsible has not been determined.

The data presented in this dissertation are the first to demonstrate an ability of central angiotensin to increase sympathetic outflow by an action at a single nucleus. The technique of microinjection represents significant advancement over intraventricular administration, which has been widely used to study the central effects of ANG II.

Although the pressor site of intraventricularly administered ANG II has been localized to the anterior third ventricle, the discrete nucleus involved is still a matter of some controversy. One drawback to intraventricular administration is the exposure of all circumventricular organs to the injectate and the possibility of activating sites also accessible to circulating agents. The technique of microinjection circumvents this problem allowing the investigator to study specific nuclei of the brain. It is also possible to study the action of a substance in areas of the central nervous system protected by the blood-brain barrier. The use of very small injection volumes decreases the spread to nearby areas making further localization of the site of action possible. It is hoped that these studies will help to advance the use of this technique.

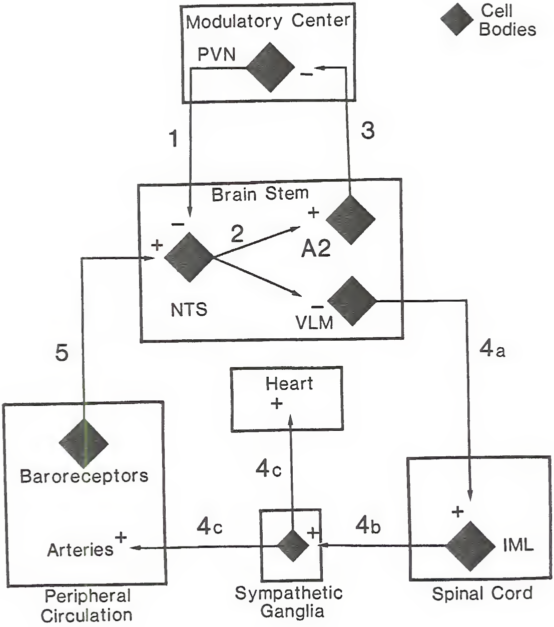
Angiotensin II has been shown in this dissertation to activate the sympathetic nervous system and yield an increase in blood pressure through an action at the NTS. In addition, a decrease in the sensitivity of baroreflexes was demonstrated independent of the ability of ANG II to increase resting arterial pressure. The hypothesis of a defective brain angiotensin system in the pathophysiology of spontaneous hypertension in rats was strengthened by the demonstration of exaggerated responsiveness to ANG II at the NTS in these rats.

The role of endogenous ANG II in the NTS of normotensive animals is not known. Presumably, tonic low-level release of ANG II within the NTS occurs and is necessary for normal functioning of the baroreflex. Microinjection of very low amounts of ANG II produced variable decreases in blood pressure suggesting that endogenous angiotensin may enhance the function of baroreflexes by decreasing the set point. At larger doses, opposite effects on baroreflexes were observed with much less variability. The action of angiotensin may represent a function not only of dose but of the physiological state of the animal. Thus, the highly variable results obtained with low doses of angiotensin may reflect individual variability of the animals, which at larger doses are overshadowed by the profound activation of sympathetic tone. Normotensive animals may be reacting to tonic release of ANG II in the NTS with what is considered normal baroreflex sensitivity and set point. Hypertensive animals, being more sensitive to ANG II, may react to tonic release of the same or slightly higher amounts of angiotensin with a depressed baroreflex and higher resting arterial pressure.

Another possibility is that the release of ANG II onto the neurons of the NTS is not tonic but occurs only when higher centers of cardiovascular control determine that baroreflex regulation of blood pressure needs to be overridden. During periods of stress or acute recovery from hemorrhage, it is desirable to increase the relative arterial pressure rapidly. Normal baroreflex function would tend to oppose this action and is therefore undesirable in certain conditions. Neuropeptide modulation of the baroreflex sensitivity and set point, by the hypothalamus in this case, represents one possible mechanism to accomplish these ends. Figure 26 represents the probable neural circuitry responsible for the actions demonstrated by angiotensin within the NTS. As shown, the NTS exerts tonic inhibition over the sympathetic nervous system presumably by inhibitory synaptic connections within portions of the ventral medulla. Ascending catecholamine fibers to the PVN allow the hypothalamus to monitor the activity of the baroreflex arc. Baroreflex afferent fibers normally excite the NTS to cause a reflex decrease in sympathetic tone. Descending peptidergic fibers from the PVN, containing angiotensin and vasopressin, directly stimulate sympathetic efferents and modulate the ability of baroreceptor afferents to initiate reflex compensation to the increased arterial pressure.

Future work in this field would ideally elucidate whether the action of ANG II on baroreflex function is tonic or phasic under normal conditions. It is also desirable to study the pattern of sympathetic activation in the

Figure 26. Schematic of potential central nervous system connections important in the NTS actions of ANG II; 1) descending hypothalamic peptide-containing pathways possibly ANG II and vasopressin; 2) brain stem interneurons projecting to several structures; 3) ascending catecholamine pathways; 4) sympathetic efferent pathways, a) bulbospinal fibers, b) preganglionic fibers, c) postganglionic fibers; 5) primary ascending baroreceptor fibers. PVN, paraventricular nucleus; A2, solitary nucleus catecholamine cell bodies; VLM, ventral lateral medulla; IML, intermediolateral cell bodies; ANG II, angiotensin II.


Modulatory Center
PVN
−
Cell Bodies
1
3
Brain Stem
+
−
+
2
A2
NTS
−
VLM
4a
5
Heart
+
Baroreceptors
4c
+
4c
+
4b
IML
Arteries
+
Peripheral Circulation
Sympathetic Ganglia
Spinal Cord

individual vascular beds to determine whether similarities exist with that seen after intraventricular or intravenous administration of angiotensin and whether the pattern is altered in the spontaneously hypertensive rat. Further studies with the NTS actions of angiotensin in other forms of experimental hypertension are important because many of these coincide with altered baroreflex function. Specifically, it may be important to determine whether long-term inhibition of angiotensin synthesis at the NTS reverses the changes in baroreflex function associated with hypertension in experimental animals as occurs with intraventricular administration of captopril.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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